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THE FUNGAL FLORA OF THE CANINE EAR

By

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INTRODUCTION

There are numerous references in the literature to the isolation of fungi from infected human ears and fifty-three different species have been reported as causal organisms of otomycosis (Wolf, 1947), the commonest being *Aspergillus, Penicillium, Mucor* and *Rhizopus*. On the other hand, a number of workers including Brown (1937) and Lumsden (1951), are of the opinion that the commonest cause of external otitis is the asporogenous yeast, *Pityrosporum ovale*.

Compared with the condition in man, little is known of the fungal flora of the infected canine ear. In a preliminary report Schoop (1951) described canine external otitis as a blastomycosis as many of the affected ears showed a profusion of "blastomycetes" to the exclusion of bacteria. A similar view was expressed by Gustafson (1954) who named the organism *Pityrosporum canis* and successfully reproduced the clinical condition in healthy dogs by instilling cultures of the yeast into the intact external acoustic meatus.

An almost identical organism, *Pityrosporum pachydermatis* Weidman, has been isolated from the external ear canals of dogs by Miss Hoven van Genderen, in Sweden (Ainsworth and Austwick, 1955).

MATERIAL AND METHODS

Material was collected by means of sterile swabs from the external acoustic meatus, the anterior nares and the tonsils of 35 clinically healthy dogs and from the external ears of 277 dogs suffering from otitis. The dogs were chosen at random, no attempt being made to select the animals according to age, sex, breed, length of coat, etc. In the case of the healthy dogs the swabs were taken within an hour of the animal's death, when the external acoustic meatus was examined for the presence of discharge, exudate or other abnormality. Cultures were discarded and the case dismissed as 'not-normal' if histological preparations subsequently showed thickening of the stratified squamous epithelium, inflammatory reaction, or alteration in the appearance or distribution of the glandular structures.

The swabs were inoculated on glucose and nutrient broth, Raulin's medium (Smith, 1946), horse blood agar, nutrient agar, and Sabouraud's maltose agar or malt agar. Incubation was at 37°C. except for duplicate cultures on Sabouraud's or malt agar which were incubated at 22°C. and one plate of nutrient agar which was incubated at 30°C. Other media used included corn-meal agar and fermentation media for yeasts (Lodder and van Rij, 1952). The above media, except where otherwise stated, were prepared according to the methods of Mackie and McCartney (1953).

RESULTS

Healthy Dogs

The incidence of fungi in the external and middle ears, the anterior nares and the tonsils of healthy dogs was as shown in Table 1. Fourteen strains of fungi were isolated from 8 (11 per cent) of the healthy external ears; four were *Aspergillus*, three *Penicillium*, two *Absidia*, three *Rhizopus*, one *Geotrichum* and one *Cladosporium*. Although fungi were present in only 4 (11 per cent) of the nares, no fewer than twelve strains were recovered representing five different genera. In addition, *Candida* species were isolated from four and six per cent of the external ears and anterior nares respectively. All middle ear cultures proved sterile and true fungi were not present in any of the tonsil swabs of the 35 healthy dogs. A finding of some interest was the presence of *Pityrosporum* species, in 36 per cent of the external ears of clinically healthy dogs, although in no case were the organisms numerous in stained films.

TABLE 1

THE FUNGAL FLORA OF HEALTHY DOGS

Tissues examined		*Fungi*		*Pityrosporum*		*Other yeasts*	
		No.	%	*No.*	%	*No.*	%
External ears	(70)	8	(11)	25	(36)	3	(4)
Middle ears	(50)	—	—	—	—	—	—
Anterior nares	(35)	4	(11)	—	—	—	—
Tonsils	(35)	—	—	—	—	2	(6)

Infected Dogs

Examination of 277 dogs with otitis resulted in the isolation of 251 strains of fungi, yeasts and yeast-like organisms from 241 (60 per cent) of the 400 affected ears. Fungi were present in 12 per cent., *Pityrosporum* in 44 per cent. and other yeasts in 6 per cent of ears. A detailed comparison of these findings with those from healthy tissues is shown in Table 2.

The results in Table 2 show that fungi, other than yeasts, were less common in the external ears of dogs suffering from otitis than they were in healthy tissues. On the other hand, although it was found that yeasts, especially *Pityrosporum*, were numerically greater in stained films of otitic material their incidence in healthy and diseased external ears appeared to be very similar.

The fact that no single strain predominated in infected ears (Table 3) also suggests that fungi are rarely of pathogenic significance in canine external otitis.

TABLE 2

THE INCIDENCE OF YEASTS AND FUNGI IN HEALTHY AND AFFECTED DOGS

Genus	*Healthy dogs (35)*				*Dogs with otitis (277)*	
	Nares (35)		*External ears (70)*		*Affected external ears (400)*	
	No.	%	*No.*	%	*No.*	%
Aspergillus	3	(9)	4	(6)	13	(3)
Penicillium	4	(11)	3	(4)	12	(3)
"Mucor Group"	2	(6)	5	(7)	13	(3)
Cladosporium	2	(6)	1	(1)	8	(2)
Geotrichum	—	—	1	(1)	3	(0·8)
Botrytis	1	(3)	—	—	1	(0·3)
Candida	—	—	3	(4)	19	(5)
Rhodotorula	—	—	—	—	5	(1)
Pityrosporum	—	—	25	(36)	177	(44)

TABLE 3

THE SPECIES OF FUNGI AND YEASTS ISOLATED FROM HEALTHY AND INFECTED EXTERNAL EARS OF DOGS

	Healthy ears (70)	*Infected ears (400)*
Absidia ramosa	1	3
Absidia corymbifera	1	1
Mucor racemosus	—	3
Rhizopus nigricans	3	4
Rhizopus species	—	2
Aspergillus fumigatus	2	7
Aspergillus terreus	1	—
Aspergillus niger	—	2
Aspergillus species	1	4
Penicillium expansum	2	4
Penicillium spinulosum	—	1
Penicillium species	1	7
Botrytis cinerea	—	1
Cladosporium herbarum	1	8
Geotrichum candidum	1	3
Candida albicans	3	11
Candida tropicalis	—	5
Candida species	—	3
Rhodotorula species	—	5
Pityrosporum species	25	177

Dermatophytes

Examination of material from healthy and infected ears, on nutrient and malt agar after incubation at 30°C. and 37°C. respectively, proved negative in every case for the presence of *Tricophyton* and *Microsporum* species.

DISCUSSION

Philips (1959) considers that otomycosis, uncomplicated by pyogenic infections, is relatively rare in dogs and that fungal infections are mostly confined to those long-eared breeds whose ears tend to stay moist when inflamed. In this present work, however, there was no apparent correlation between the fungal flora of affected ears and the fact that nearly one third of the dogs suffering from otitis were Spaniels; nor were the findings related to the age and sex of the animals examined.

The commonest fungus in animal infections is *Aspergillus fumigatus*, a saprophytic mould which may become a virulent and lethal pathogen in certain circumstances (Ainsworth, 1954). Not only was this species more frequent in the healthy auditory meatus, but the fact that it was isolated from only 2 per cent of the infected ears suggested that its presence in cases of canine otitis was merely the result of contamination. In no case did the clinical picture or the degree of growth of primary cultures suggest that *A. fumigatus* was of aetiological significance in canine otitis. The same was probably true of other species of *Aspergillus* and of *Mucor, Absidia, Rhizopus, Cladosporium, Geotrichum* and *Botrytis*.

The yeasts, however, may be of greater importance. Although *Candida* species were isolated from less than five per cent of the healthy and affected ears, three cases of acute mycotic otitis were encountered during the survey one of which was caused by *C. albicans* and the other two by *C. tropicalis*. All were encountered in animals which had received prolonged antibiotic therapy, the *C. tropicalis* infections being secondary to otitis associated with coagulase positive staphylococci while the other was secondary to a *Pseudomonas aeruginosa* infection. These findings are similar to those of Duncan (1945), Woods (1951), Moore (1951) and Huppert, McPherson and Cozin (1952) who noted cases of acute mycotic otitis in man and demonstrated the importance of yeasts as a cause of certain side effects during treatment with antibiotics.

The part played by *Pityrosporum* species in initiating infection is not obvious as their incidence in the external ear canals of both healthy and affected dogs is very similar. These asporogenous yeasts appear to be closely related to *P. ovale* of human origin which by invading the epidermis, is said to cause multiplication of the cells with a consequent scaling of the horny layer and which has also frequently been described as a cause of seborrhoea, dandruff and other skin conditions in man. Although the dog strains differed

from those from human sources by growing freely on the surface of Sabouraud's maltose or glucose agar without added oleic acid or similar substances, they may, nevertheless, give rise to an inflammatory reaction within the external acoustic meatus.

CONCLUSIONS

Moulds were comparatively rare in the external ears of healthy dogs and of dogs suffering from otitis.

The commonest species, *Aspergillus, Penicillium, Absidia* and *Rhizopus,* were equally prevalent in healthy and affected ears and were probably of no pathological importance.

Candida species occasionally gave rise to acute mycotic otitis as a result of prolonged antibiotic therapy.

Pityrosporum species may be of aetiological significance in otitis by causing irritation of the epithelium of the external acoustic meatus, but they were also frequently present in healthy dogs.

Dermatophytes were not recovered from the ears of 35 clinically healthy dogs or of 277 dogs suffering from external otitis.

ACKNOWLEDGMENTS

The writer is indebted to Dr. A. Wilson Taylor for his help and advice, and to Mr. A. R. Withers, Mr. W. W. Gregor and the staff of the Small Animal Clinic of this School for their co-operation in supplying material.

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THE EXPERIMENTAL INFECTION OF CATTLE WITH *MYCOBACTERIUM JOHNEI*

II. Adult Cattle Inoculated Intravenously

By

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INTRODUCTION

In a previous paper (Rankin, 1958a) it was confirmed that Johne's disease could be successfully transmitted in cattle by the intravenous inoculation of calves with *Mycobacterium johnei*. Taylor (1953) has expressed the opinion that if calves were over six months of age when inoculated, Johne's disease would not result. Only two such animals were used in Taylor's experiment and the present paper describes a further investigation of this age/susceptibility relationship using mature cattle in greater number.

MATERIALS AND METHODS

Mycobacterium johnei. The strain of *M. johnei* was that used to infect the calves in the previous experiment (Rankin, 1958a) and the inoculations were made at the same time and from the same batch of suspension as that used for the calves.

Experimental animals. Six three-year-old Ayrshire cows were used. They had been placed in the isolation units at the Field Station when one or two weeks of age and had been kept there ever since. At the time of inoculation they were either lactating or dry and in-calf. At the routine annual tuberculin tests none of the cattle had ever shown any reaction to either avian or mammalian tuberculin and they were still negative at the time of inoculation. After inoculation the cattle continued to be kept in an isolation unit, exercise being taken in an enclosed yard with a concrete floor.

Maintenance of animals and cultivation of the organism. The maintenance of the animals and methods of recovering *M. johnei* from tissues and faeces are described in the first paper of this series (Rankin, 1958a).

Method of infection. Each of the six cows was inoculated intravenously with 100 mg. wet weight of *M. johnei* suspended in 10 ml. Ringer's solution. They were kept together as a group. Faeces samples were collected at monthly intervals and examined culturally for the presence of *M. johnei*. Four years after inoculation the cattle were killed and tissues were examined macroscopically, microscopically and culturally for Johne's disease, and/or the presence of *M. johnei*.

RESULTS

No immediate effect of the inoculation was observed. The cows behaved and fed in a normal manner and those in-milk continued to yield at their normal level.

Excretion of M. johnei

Samples of faeces were subjected to cultural examinations at monthly intervals over a period of 47 months. At no time was *M. johnei* recovered from the faeces of any cow.

Clinical Observations and Post-mortem Findings

During the experiment no cow showed any symptom indicative of Johne's disease.

One cow (6) developed an acute staphylococcal mastitis and was slaughtered on humanitarian grounds 21 months after the beginning of the experiment. When the five remaining cows were slaughtered 4 to 4½ years after inoculation four failed to show any macroscopic or microscopic lesion, of Johne's disease in the intestine. One animal (2), however, showed very slight thickening and corrugation of the mucous membrane of the ileum (Fig. 1). These slight signs were not regarded as being diagnostic of Johne's disease at that time but, on histological examination, scattered endothelioid cells and a few giant cells were seen (Fig. 2). Two acid-fast bacilli were seen in one of the giant cells. No other acid fast bacilli were found. *M. johnei* was recovered culturally from each of the five cows at the post-mortem examination. The results of these cultural tests are shown in Table 1.

TABLE 1

RESULTS OF CULTURAL EXAMINATIONS OF VARIOUS ORGANS
7-year-old cows which had been inoculated intravenously with 100 mg. *M. johnei* at 3 years of age

Site cultured	*Cow number*					
	1	*2*	*3*	*4*	*5*	*6*
Duodenum	−	+	−	−	−	Died as a result of intercurrent infection
Ileum	−	+	−	−	−	
Caecum	−	+	−	−	−	
Rectum	−	+	−	−	−	
Uterus	−	−	−	−	−	
Ovary	−	−	−	−	−	
Bladder	−	−	−	−	+	
Udder	−	−	−	−	−	
Lung	−	−	−	−	−	
Kidney	−	−	−	−	−	
Liver	−	−	−	−	−	
Spleen	−	−	−	−	−	
Lymph nodes						
Submaxillary	−	−	−	−	−	
Retropharyngeal	+	−	−	+	+	
Prescapular	−	−	−	−	−	
Popliteal	−	−	−	−	−	
Iliac	−	−	−	−	−	
Hepatic	−	+	−	−	−	
Mediastinal	−	−	−	+	−	
Ileo-caecal	−	+	+	+	−	
Mesenteric	−	+	−	−	−	
Supramammary	−	+	−	−	−	

DISCUSSION

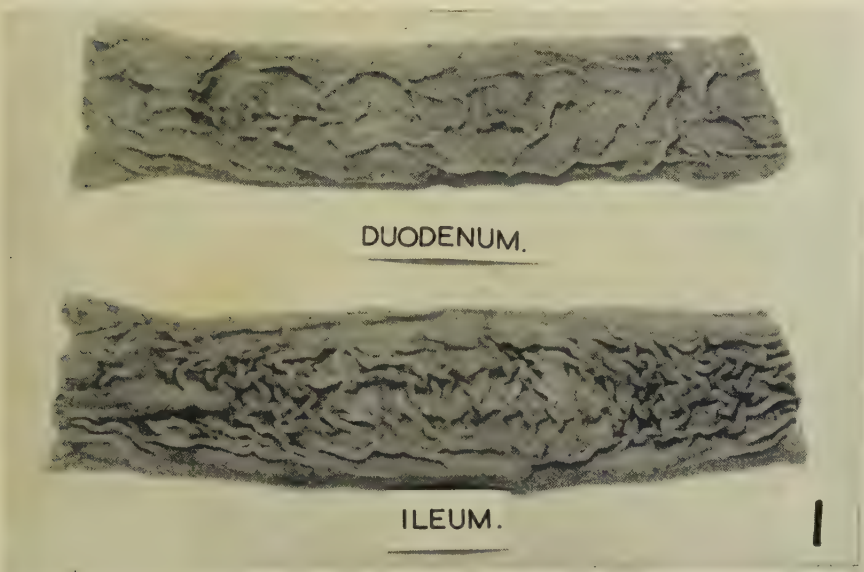
In the first experiment in this series (Rankin, 1958a), four out of six one-month-old calves inoculated intravenously with 100 mg. *M. johnei* developed clinical Johne's disease within 4 years. In the present experiment none of five adult cattle similarly inoculated with the same strain at the same time developed clinical Johne's disease. Further evidence is thus added to support the widely held opinion that in this disease there is a difference in susceptibility between the young and the old animal.

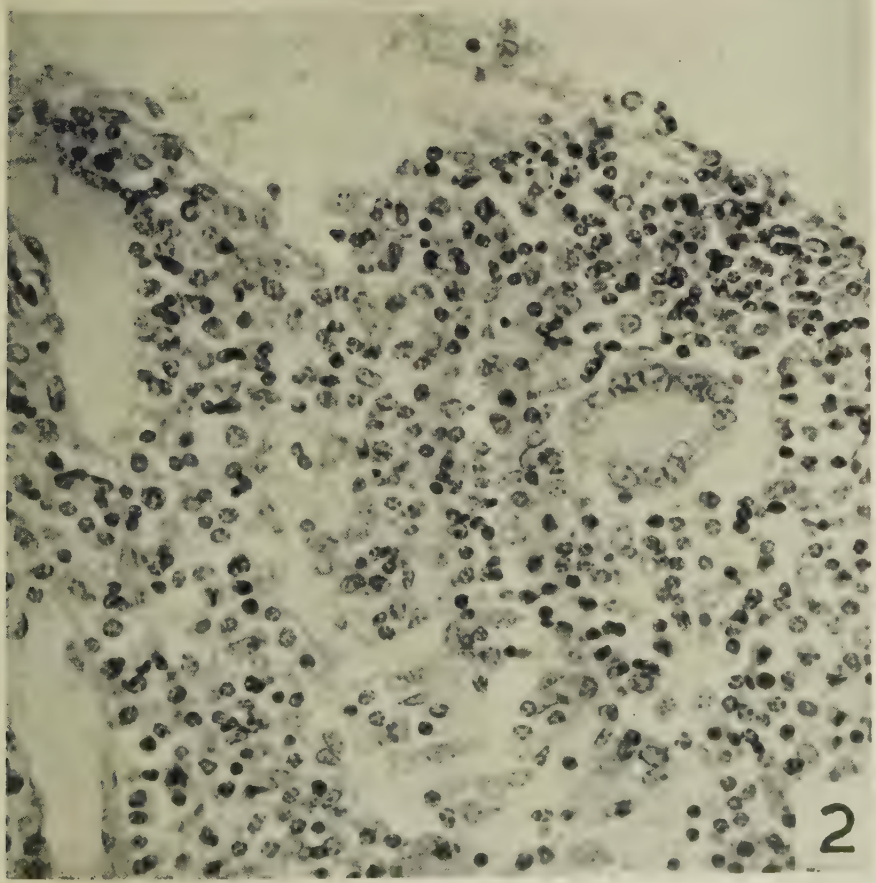
One of these adult cows did show microscopical lesions consistent with a diagnosis of Johne's disease, but she was in excellent condition when killed, and regular cultural examination of her faeces throughout the previous four years had given consistently negative results. Although it is impossible to predict accurately what the outcome would have been had this cow been allowed to live for a few more years it is considered that there are grounds for supporting the view that the infection would have regressed. This opinion is based on the ill-defined nature of the lesion, the inability to recover the organism from the faeces, and the fact that in the previous experiment in calves *M. johnei* was recovered more or less regularly for 15 months from the faeces of two animals which, on being killed 3½ years later failed to show lesions in the intestine. It is believed that, as the recovery of organisms was so regular, these two cattle must have had, at one time, lesions much more severe than those in the cow under consideration.

The reason for this apparent resistance of cattle to Johne's disease when exposed to infection with *M. johnei* for the first time as an adult is not at this stage clear. That it is simply the effect of an antibody resulting from previous contact with the specific, or an allied antigen, does not seem likely in this case. All the cattle in the experiment were brought to the isolation compound at this laboratory within a short time of birth and had been maintained there for the 3 years prior to the start of the experiment. During that time these cattle had been subjected to four tuberculin tests at none of which did any of them react to either avian or mammalian tuberculin.

It is known (Rankin, 1958b) that the adult rabbit can also resist attempts to produce Johne's disease in this way. As far as is known the disease does not occur naturally in the rabbit and this resistance has in the past been regarded as a species resistance. If, however, very young rabbits are inoculated Johne's disease can be induced.

The very serious nature of tuberculosis contracted during the first six months of human infant life is well recognised but not understood. Recently Lurie (1955) has drawn attention to the correlation between the activity of certain hormones and the susceptibility, or resistance, of a host to the invasion of the tubercle bacillus. A similar approach to the question of age resistance in Johne's disease might yield valuable information.





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CONCLUSIONS

None of five cows inoculated intravenously when 3 years old with *Mycobacterium johnei* developed clinical Johne's disease within 4 years.

Microscopical lesions of Johne's disease were found in the intestine of one of the cows at post-mortem examination.

Mycobacterium johnei was recovered culturally from the lymphatic system of all five cows 4 years after inoculation.

The question of a resistance which is apparently associated with age is discussed.

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LEGENDS TO ILLUSTRATIONS

Fig. 1 Portions of the intestine of cow No. 2 illustrating the degree of thickening and corrugation of mucous membrane encountered.

Fig. 2 Section of the ileum of cow No. 2 showing slight accumulation of endothelioid cells and one giant cell in the tip of a villus. Two acid-fast bacilli were seen in this giant cell.
H. & E. x 320

THE EXPERIMENTAL INFECTION OF CATTLE WITH *MYCOBACTERIUM JOHNEI*

III. Calves Maintained in an Infectious Environment

By

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INTRODUCTION

Two previous papers in this series describe the infection of calves (Rankin, 1958) and adult cattle (Rankin, 1961) by the intravenous route with *Mycobacterium johnei*. Those infected as calves developed clinical Johne's disease whereas those infected as adults did not. Taylor (1953) has suggested that this age resistance to clinical breakdown is present by the time the calf is six months old. This would obviously be of great practical importance in controlling the disease and it was therefore decided to test the hypothesis by exposing calves of varying ages to an infectious environment.

MATERIALS AND METHODS

Experimental animals. Nine Ayrshire heifer calves of various ages born at the Field Station from cows known to be free from Johne's disease were used. They were reared and maintained as a group in an Isolation Unit in a similar manner to those in the previous experiments (Rankin, 1958).

Donor animals. Three similar one-month-old calves were inoculated intravenously with 100 mg. wet/weight of a culture of *M. johnei*. The strain used was the one used in the previous infection experiments and had been re-isolated post-mortem from one of the animals. The three infected calves were kept with the experimental animals and their faeces were the source of infection for the creation of the necessary infectious environment. All three calves began excreting *M. johnei*. in their faeces within two months of infection, and all became clinical cases of Johne's disease within two years.

Maintenance. The feeding and general husbandry of the animals were as described in the first paper in this series (Rankin, 1958).

Cultural techniques. The methods of recovering *M. johnei* from tissues and faeces are described in the first paper of this series (Rankin, 1958).

Conduct of experiment. The three infected donor calves were maintained on straw in the covered yard of an Isolation Unit. Nine calves were introduced to this environment, three at one week, three at one month and three at six months of age. Continuous contact was maintained between all groups until the donors died from clinical Johne's disease two years after the beginning of the experiment.

Faeces samples were cultured for the presence of *M. johnei* at monthly intervals for more than three years, and the animals were killed and subjected to post-mortem examination either when they developed clinical Johne's disease or during their fifth year of life.

RESULTS

Faeces Cultures

M. johnei was recovered in culture from the faeces of all nine animals in the experiment (Table 1). One calf gave a positive faeces sample one month after contact with the environment, but generally it was three to five months before the faeces became positive. Cow 2 gave only one positive sample (sixteen months after contact) during the three years of the experiment. Positive samples were found much less frequently in these contact animals than was the case in the calves infected intravenously (Rankin, 1958), positive samples being frequently followed by a long series of negative samples.

The record of animal 5 is particularly interesting. After giving ten consecutive positive faeces samples between the fifth and sixteenth month of contact, her faeces became negative and remained so until the end of the test period. When she was killed after four and a half years, no macroscopic lesion of Johne's disease could be found and there appeared to be strong circumstantial evidence that this animal recovered from an infection contracted during the first year of life. Extensive histological examination of the bowel, however, revealed definite lesions of Johne's disease.

Clinical Picture

Six of the nine calves placed in contact with the infectious environment developed Johne's disease. Four of these were typical clinical cases, but in the other two the disease was only revealed after a careful post-mortem examination. Cases were found in all three age groups; two (7 & 9) in the animals placed in contact at six months of age, three (4, 5 & 6) in those placed in contact at one month of age and one (1) in those placed in contact at one week of age. One cow (4) developed clinical disease and had to be killed during her first lactation; all the others were killed during their second lactation when they were about four years old.

The milk yields during the first lactation and up to the time of death in the second lactation are shown in Table 2. The animals which eventually became clinical cases appear to have given a poor yield in their first lactation but the two sub-clinical cases are well up to the mean yield of twenty similar cows kept under similar conditions.

Post-mortem Results

The four clinical cases (4, 6, 7 & 9) showed severe typical lesions of Johne's disease throughout the bowel. In one case (1), the lesions were much less extensive, and both the thickening and the corrugation of the mucous membrane were less marked. In the remaining case (5), there were no macroscopic changes, but definite lesions consisting of endothelioid cells and giant cells containing acid-fast bacilli were seen microscopically in sections of the terminal portion of the ileum.

TABLE 1

RESULTS OF MONTHLY CULTURAL EXAMINATIONS OF FAECES SAMPLES

Cattle placed in contact with an environment contaminated with *Mycobacterium johnei*

Ref. No.	*Age when expd.*	*Months after exposure* 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48	*Post mortem findings*
1	1 week	− − + C − + − − − − − − − − C + − − − − − − − − − − − + − + − − − − + + + + − − K	Sub-clinical
2	,,	− − − − − − − − − − − − − − C + − − − − − − − − − − − − − − − − C − − − − − − − − K	No disease
3	,,	− − + + + − − − − − − − − − C − − − − − − − − − − − − − − − − − − − + − − − − K	No disease
4	1 month	− − + + + − + C + − − + + − − + − − − C + − + + − − − − + + + + + K	Clinical
5	,,	− − − + + + + + + + + + − − − − − − − C − − − − − − − − − − − − − − − − − − −

TABLE 2

LACTATION OF COWS WHICH, AS CALVES, HAD BEEN PLACED IN AN ENVIRONMENT WITH *MYCOBACTERIUM JOHNEI*

Reference No.	*Age when exposed*	*1st lactation*		*2nd lactation*		*Johne's disease status*
		Days	*Gallons*	*Days*	*Gallons*	
1	1 week	271	650	175	470	Sub-clinical
2	,,	270	510	—	—	No disease
3	,,	304	690	161	450	No disease
4	1 month	30	60	—	—	Clinical
5	,,	294	640	182	580	Sub-clinical
6	,,	253	400	81	60	Clinical
7	6 months	257	400	85	90	Clinical
8	,,	Infertile		Infertile		No disease
9	,,	165	270	54	90	Clinical

The mean yield of twenty cows kept under similar conditions of management was 680 gallons for a lactation of 305 days.

TABLE 3

RESULTS OF POST-MORTEM CULTURAL EXAMINATIONS OF VARIOUS ORGANS

Cattle reared and maintained in an environment contaminated with *M. johnei*

Site cultured	*Exposed to contaminated environment at age of:*								
	1 week			*1 month*			*6 months*		
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Lung	—	—	—	+	—		—	—	+
Kidney	—	—	—	+	—		—	—	+
Liver	+	—	—	+	—		—	—	+
Spleen	—	—	—	—	—		—	—	+
Duodenum	+	—	+	+	—	+	—	—	+
Ileum	+	—	—	+	+	+	+	+	+
Caecum	+	—	+	+	—	+	+	—	+
Rectum	+	—	+		+	+	+	—	—
Bladder	—	—	—		—		—	—	—
Uterus			+	+	+	+	—	—	+
Ovary	+		—	+	—			—	+
Udder	—	—	—		—	+			+
Lymph nodes:									
Submaxillary	+	—	—	+	—		—	+	+
Retropharyngeal	+	+	+	+	+		+	+	+
Prescapular	+	—	—	+	—		—	—	+
Popliteal	—	—	—	+	—		—	—	+
Iliac	+	—	—	+	—		—	—	+
Hepatic	+	—	+	+	+		+	—	+
Mediastinal	+	—	+	—	—		+	+	+
Ileo-caecal	+	+	+	+	+	+	+	—	+
Mesenteric	+	—	—	+	—	+	+	+	+
Supramammary	+	—	—	+	—		—		+

In all cases the gross diagnosis was confirmed histologically and by demonstration of acid fast bacilli in smears of the intestinal mucosa. Cultural examinations of the viscera and lymph nodes were also carried out and the results are tabulated in Table 3.

DISCUSSION

Since two of the three cattle exposed to the infection for the first time at six months of age developed Johne's disease in clinical form, the premise that six months after birth calves have already developed a natural immunity to Johne's disease does not hold good. It may be that continuous exposure to small doses of bacilli over a long period is a more severe test than the single large intravenous dose administered by Taylor (1953) or perhaps Taylor's two calves would not have developed the disease whatever the age at which they were exposed. For example, two of the animals in this experiment did not develop Johne's disease although they were first exposed at one week old. That they did become infected is not in doubt as the bacillus was recovered from the lymphatic system of each at post-mortem. Whatever the explanation, it would certainly be unwise to assume that calves are resistant to Johne's disease by the time they are six months old. That a resistance does develop with age is not here in dispute, but it probably takes longer to appear than has so far been thought. Calves exposed to infection in this way took longer to show symptoms of the disease than did the calves infected intravenously (Rankin, 1958), but the stunting of growth caused by the intravenous method was not seen here, the clinical cases reaching normal stature before breakdown occurred. Milk yield, however, was affected in the lactation prior to breakdown.

It is interesting to note that all the animals exposed to the environment become infected with *M. johnei*. They all became sensitive to avian tuberculin within three months of exposure, and the organism was cultivated from both diseased and non-diseased animals four years after first exposure.

M. johnei does not seem to be a particularly aggressive pathogen, for, although Johne's disease is widespread throughout the country, the death rate from the disease in diary cattle is under 1 per cent. Also in few experimental infections do 100 per cent of the infected animals contract the disease despite very heavy dosing. It is gratifying therefore to find that in this experiment 66 per cent of the animals at risk contracted the disease. For experiments on prophylaxis where it might be felt that experimental challenge by the intravenous route might overwhelm an immunity which would prove to be adequate under field conditions, challenge by contact would appear to be a reasonable alternative.

CONCLUSIONS

Six of nine cows which had been continuously exposed since calf-

hood to an environment naturally contaminated with *Mycobacterium johnei* developed Johne's disease.

Two of the six cows were six months old when first introduced to the infected environment.

When the cows were killed at up to $4\frac{1}{2}$ years after exposure *Mycobacterium johnei* was cultivated from all nine, usually from a wide variety of sites.

The cows which became clinical cases gave a low milk yield in the lactation prior to the appearance of the disease.

Exposure to a deliberately created infectious environment would seem to be a useful experimental procedure until a more reliable and acceptable method of experimental infection becomes available.

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UTERINE PATHOLOGY OF TEMPORARILY-FASTED PREGNANT MICE

By

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INTRODUCTION

Although it has been known for many years that pregnancy in many species of animals is often terminated by the death of ova or embryos (Meyer, 1917; Corner, 1923), it was not until comparatively recently that early embryonic mortality was shown to be involved in the pathogenesis of some types of infertility in cattle and sheep (Laing, 1949; Tanabe and Casida, 1949; Dutt, 1954; Hawk, Wiltbank, Kidder and Casida, 1955; Inkster, 1959). Little is known of the causes in these species, except, that in cattle, uterine infections such as vibriosis are apparently sometimes involved. There is an increasing amount of evidence that minor nutritional deficiencies or stresses are involved in infertility in these animals (Hignett, 1956; McClure, unpublished data).

In the hope of understanding more about early embryonic mortality of these non-infectious causes in cattle and sheep, a study was made of the morbid anatomy associated with the infertility in female mice caused by fasting for one or two 48 hour periods during early pregnancy. This was considered to involve the death of the young embryos and was described in earlier papers (McClure, 1958, 1959).

MATERIALS AND METHODS

Virgin white mice, eight to ten weeks of age, from a closed colony were used for the experiments. The mice were fed on a standard pellet diet which had proved adequate for normal growth and reproduction, and were kept in a room maintained at 18 to 24 °C. Water was always freely available.

Fifty-three female mice were mated in separate cages. The mice were examined once daily for copulation plugs, the occurrence of which was taken as evidence of mating and used for determining the stage of gestation. Subsequently they were fasted for one 48 hour period or two 48 hour periods separated by a 48 hour period of feeding ad libitum. The first period of starvation started two, three or four days after the detection of the copulation plugs. Nineteen female mice were similarly mated, but were not subsequently fasted and acted as controls.

The mice from both the treated and control groups were killed from three to 13 days after the detection of plugs and their uteri and contents examined to assess the state of pregnancy. At autopsy on the third day the fallopian tubes were teased in, and the uterine horns flushed with 0·9 per cent sodium chloride solution which was then searched under the microscope for ova, morulae and blastocysts. On the fourth and fifth days the fallopian tubes and one uterine horn were washed and the other terine horn fixed in a ten per cent formalin solution and examined

histologically. After the fifth day the whole uterus was fixed and examined histologically, except when the uterus appeared to be non-gravid it was flushed and the contents examined microscopically.

RESULTS

Of the 19 control mice 16 (84 per cent) were pregnant and appeared to be normal in all respects, and three (16 per cent) were neither pregnant nor showed any signs of having been pregnant.

Twelve mice were examined during the first 48 hour period of fasting. Ten (83 per cent) were pregnant and normal and two (17 per cent) were not pregnant. Fifteen mice were examined during the 48 hour period immediately following the end of the first period of fasting. Two (13 per cent) were pregnant and normal, six (40 per cent.) were pregnant but the deciduomata or deciduomata and embryos were degenerating and seven (47 per cent) were neither pregnant nor showed evidence of pregnancy. Of these 15 mice, two were fasted from the end of the second day after finding copulation plugs, seven from the end of the third and six from the end of the fourth day. In the first group when examined 24 hours after the end of fasting one mouse was pregnant and normal, the other not pregnant. In the second group 24 hours after the end of fasting the deciduomata or embryos and deciduomata of all three mice examined were degenerating. Forty-eight hours after the end of fasting the deciduomata and embryos of one mouse were degenerating and the uteri of three mice were now empty. In the third group one mouse was pregnant and normal, two contained degenerating deciduomata and embryos and three were not pregnant.

Ten mice were examined during the second 48 hour period of fasting. Two (20 per cent) were pregnant and normal, four (40 per cent) were pregnant but the deciduomata or embryos and deciduomata were degenerating and four (40 per cent) were neither pregnant nor showed evidence of pregnancy. Sixteen mice were examined in the 96 hour period after the end of the second 48 hour period of fasting. None was either pregnant or showed evidence of pregnancy.

Description of the Lesions

Lesions were not seen until the day following the end of the first 48 hour period of fasting. The first detected sign of abnormality was the occurrence of inter-cellular haemorrhage close to the periphery of the unattached parts of the deciduomata. It appeared that blood then leaked out into the lumen of the uterus between the deciduomata. Subsequently the cells of the deciduomata and embryos became necrotic and the tissue was invaded by leucocytes, mainly polymorphonuclear. Finally the deciduomata liquefied and the debris was absorbed. Occasionally blood and debris were discharged through the cervix. Photomicrographs of normal and degenerating deciduomata are shown in Figs 1 to 6.

DISCUSSION

The results of the experiment indicated that fasting female mice for as little as 48 hours terminated pregnancy within a few hours after the end of the fasting period. Although the number of animals examined is small, the postmortem results support the earlier experiments (McClure, 1959) in which a single 48 hour period of fasting starting at the end of the fourth day after oestrus (or third day after the detection of the copulation plug) did not allow any mouse to litter. In the present experiment, following an equivalent fast 3/3 pregnancies were terminating when the mice were examined 24 hours after the end of the fast. When examined 48 hours after the end of the fast, in 1/4 the deciduomata and embryos were degenerating and in 3/4 the uteri were empty.

There was no evidence of embryonic death occurring in the 12 mice killed during the 48-hour period of starvation. In this group the proportion of mice which were pregnant was similar to that of the control mice. Starvation for 48 hours starting from the second or fourth day after mating did not appear to be as effective as from the third day for, when examined during the 48-hour period after the end of fasting two of the eight mice involved contained normal embryos. Again, this agrees with the results of the earlier experiment. After the second period of fasting none of the 16 mice in the group was pregnant.

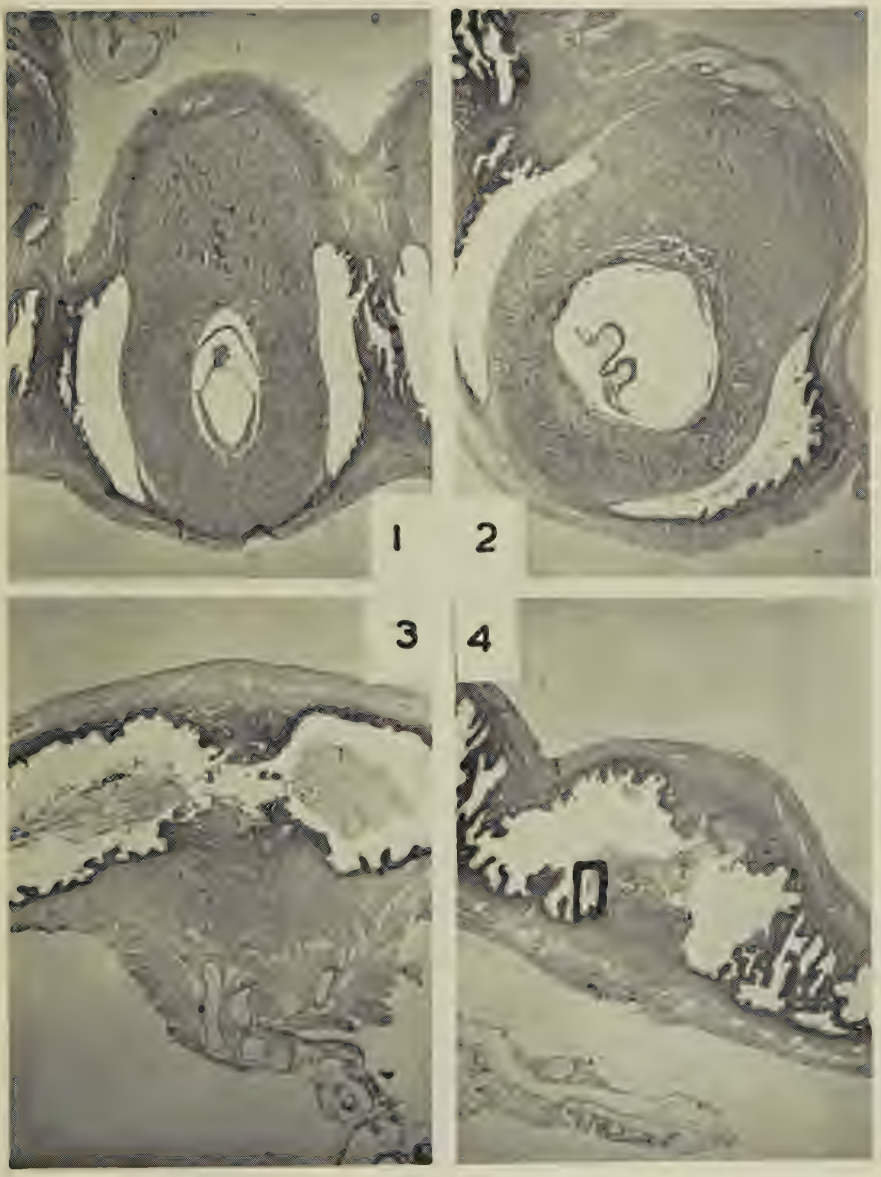
The primary lesion appeared to be in the deciduomata and the embryo appeared to die after this lesion had occurred. This suggests that the pathogenesis involves hormonal factors causing or allowing the failure of the deciduoma and consequently interference with the nutrition of the embryo. Subsequent work in which the pregnancies were protected by injections of progesterone and/or chorionic gonadotrophin has supported this suggestion (McClure, 1960).

CONCLUSIONS

White mice were fasted for one or two 48 hour periods commencing on the second, third or fourth days after mating. The fasting caused haemorrhage and necrosis of the deciduomata, death of the embryos, leucocytic invasion, liquifaction and absorption of the debris. When the fasting started on the third day lesions were present in all the mice examined one day after the end of fasting, and most of the debris had been absorbed by the end of the second day. The initial lesion was haemorrhage in and from the deciduomata.

ACKNOWLEDGMENTS

The sections were cut and stained by Mr. J. Kerr, photographic film was developed and printed by Mr. D. Macqueen and Mrs. S. Fry assisted in the care of the mice.



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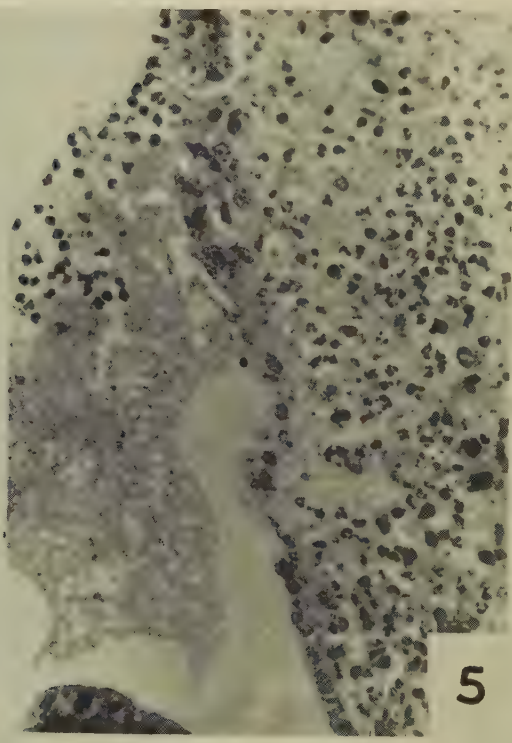

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Normal deciduoma and embryo from mouse, eight days after finding copulation plug. H & E, × 25.

Fig. 2. Early stage of degeneration of deciduoma with normal embryo, from mouse eight days after finding copulation plug. Mouse was starved CP +2 to 4 and CP +6 to 8 days. Inter-cellular haemorrhage is present, particularly at the periphery of the unattached parts of the deciduoma and polymorphonuclear leucocytes and free erythrocytes are in lumen of uterus. H & E, ×25.

Fig. 3. Advanced stage of degeneration of deciduoma and presumably embryo from mouse, eight days after finding of copulation plug. Mouse was starved CP +2 to 4 and CP +6 to 8 days. Most of the deciduoma has disintegrated and debris contains many polymorphonuclear leucocytes. H & E, × 25.

Fig. 4. Advanced stage of degeneration of deciduoma and presumably embryo from mouse, six days after finding of copulation plug. Mouse was starved CP +3 to CP +5 days. Cells of deciduoma are necrotic and deciduoma has been invaded by many polymorphonuclear leucocytes. H & E, × 25.

Fig. 5. Enlargement of part of the field in plate 4 showing polymorphonuclear leucocytes and debris. H & E × 400.

Fig. 6. Uteri of normal 8-day pregnant mouse, lower and, upper, a mouse of the same stage of gestation containing degenerating deciduomata and embryos. A section of this uterus is shown in Fig. 3.

POSTMORTEM FINDINGS IN AVIAN OSTEOPETROSIS

By

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INTRODUCTION

Jungherr and Landauer (1938) in their original work on osteopetrosis commented on the frequent association of the bone lesions with lymphomatosis and from transmission studies concluded that avian osteopetrosis is caused by an agent that carries both osteogenetic and lymphogenetic properties. A similar experimental observation by Brandley, Nelson and Cottral (1942) led them to consider the possibility of a single etiological factor. The virtual absence of osteopetrosis in their control birds, however, in which the incidence of lymphomatosis was 21·2 per cent., caused them to decide that an additional factor was involved in the appearance of osteopetrosis. In 1947 Burmester and Cottral reported a high incidence of osteopetrosis after injection of tumour filtrates or plasma from birds with visceral tumours. In transmission experiments with strain RPL 12, Burmester, Prickett and Belding (1946) noted the development of osteopetrosis and lymphomatosis using donors with either extensive osteopetrosis and no visceral lesions or with widespread visceral involvement and no bony changes. Again with strain RPL 12, Gross, Burmester and Walter (1959) found osteopetrosis coexisting with erythroleucosis, visceral lymphomatosis and haemangiomatosis. Other RPL strains have also produced osteopetrosis in susceptible recipients (Burmester, 1947).

It is not surprising therefore that osteopetrosis has been considered by numerous workers as one part of the avian leucosis complex (Jungherr, Doyle and Johnson, 1941; Cottral, 1952; Beard, 1957), although the dissimilarity between the bone lesions and the various soft tissue changes in the leucosis complex has often caused doubts as to the justification of including osteopetrosis in this syndrome.

This paper describes the soft tissue changes found on postmortem examination of birds affected with osteopetrosis in which the disease was experimentally induced as described previously (Holmes, 1958, 1959). The observations are compared with those in birds exposed to infection which failed to develop bone lesions and also with uninjected controls. The possible association of osteopetrosis with the leucosis complex is considered in relation to these findings.

MATERIALS AND METHODS

Observations are recorded on 108 birds (83 males and 25 females) which developed osteopetrosis and the postmortem findings are compared with those in 119 (69 male and 50 female) control birds and 190 (138 male and 52 female) inoculated chicks which did not develop any bone lesions. The 108 osteopetrosis cases ranged in age from 5 to 97 weeks. Twenty-eight of these birds were killed and 80 of them died. In the great majority of affected birds osteopetrosis had been induced by the intraperitoneal injection into day old chicks of whole blood from active cases of the disease. Others developed following the use of unfiltered plasma intra-

peritoneally and by mouth, filtered plasma or bone marrow intraperitoneally and in a few cases bone marrow was injected into the lower end of the tibia at one day old. These were the experimental methods used to produce the disease in 100 of the birds in this study (Fig. 1). Of the remaining 8 cases of osteopetrosis 5 were the progeny of affected birds and 3 developed after injection of blood into the amnion of the developing embryo.

Postmortem examination comprised inspection of the viscera, routine histological examination of liver, kidney and spleen and if other lesions were present these were also examined microscopically. Sections were stained by Haematoxylin and Eosin and, where necessary, P.A.S. and Green Trichrome were also used.

RESULTS

The postmortem findings in 108 birds with osteopetrosis are summarised in Table 1. The term "neoplasia" includes the leucosis complex as well as specific tumours. Table 2 shows the postmortem changes in 190 birds which were inoculated but failed to develop osteopetrosis and in 119 controls which were not injected.

TABLE 1

SUMMARY OF P.M. FINDINGS IN BIRDS WITH EXPERIMENTALLY INDUCED OSTEOPETROSIS*

Age (wks.)	*No. of birds*	*Number of birds*		
		Osteo only	*Osteo + neoplasia*	*Osteo + other lesions*
5–20	43	36(83·7%)	4(9·3%)	3(7·0%)
21–40	46	31(67·4%)	14(30·4%)	1(2·2%)
46–100	19	12(63·1%)	4(21·0%)	3(15·9%)
TOTAL	108	79(73·1%)	22(20·4%)	7(6·5%)

*Produced by injection of whole blood, plasma or bone marrow intraperitoneally at 1 day old from active cases. This table also includes 5 cases which were the progeny of affected birds and 3 following intramnion inoculation as 8 – 12 day embryos.

Tumours were present in 22 birds with osteopetrosis, in 9 injected birds which remained skeletally normal and in 2 controls. These are classified in Table 3. The tumours in the osteopetrosis cases appeared irregularly throughout all seven passages under consideration and were not associated with any particular donor. (Fig. 1). During this period 13 donors were used. Of these, one of 6 birds used for the second passage had a fibrosarcoma in a kidney, but this bird and the osteopetrosis cases resulting from it were not used for further transmissions. The donors for direct transmission from the first to the seventh passage were all normal both macroscopically and microscopically, except for the presence of typical bone lesions of osteopetrosis.

TABLE 2

SUMMARY OF P.M. FINDINGS IN INJECTED BIRDS* WHICH DID NOT DEVELOP OSTEOPETROSIS AND IN CONTROL (UNINJECTED) BIRDS

	Age (wks.)	*No. of birds*	*Number of birds*	
			Normal	*With neoplasia*
Injected Birds Osteo -ve	5–20	16	15(94·0%)	1(6·0%)
	21–40	170	166(95·3%)	8(4·7)%
	46–100	4		
	Total	190	181(95·3%)	9(4·7%)
Controls	5–20	4	117(98·3%)	2(1·7%)
	21–40	115		

* Injected intraperitoneally at 1 day old with whole blood, plasma or bone marrow from active cases of Osteopetrosis.

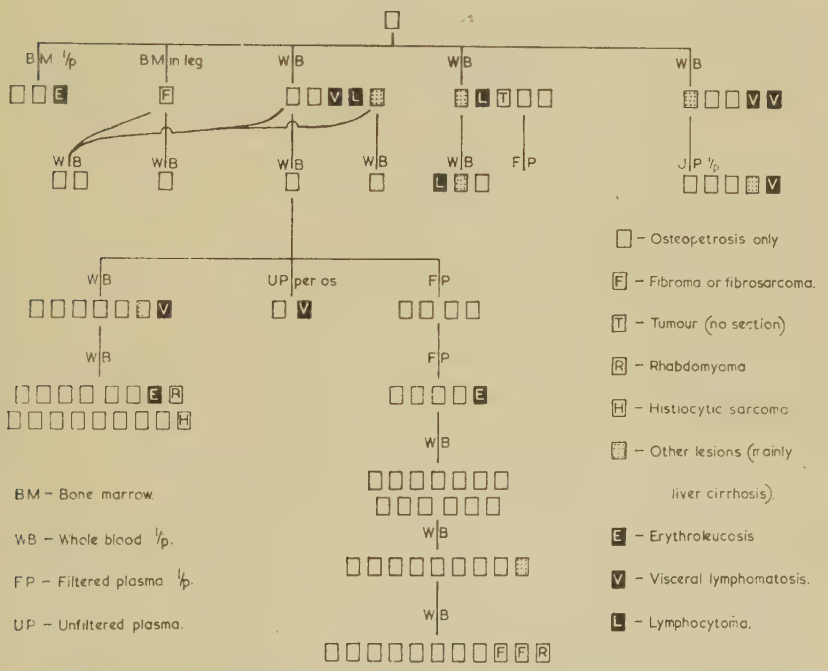
TABLE 3

TYPES OF TUMOURS FOUND ON POSTMORTEM

	Injected birds		*Uninjected controls*
	Osteo +ve	*Osteo —ve*	
No. of birds examined	108	190	119
Visceral lymphomatosis	7	2	1
Erythroleucosis	3		
Lymphocytomata	3	3	
Fibrosarcomata	3		
Rhabdomyomata	2		1
Fibromata	1	2	
Histiocytic sarcoma	1		
Embryonal nephroma	1		
Tumour (no sections)	1		
Haemangio-endothelioma		1	
Adenoma		1	
Total	22	9	2
% of birds with tumours	20·4	4·7	1·7

At the first passage 8 birds out of 19 with osteopetrosis had neoplasia, 2 out of 13 in the second, 2 out of 13 in the third, 4 out of 22 in the fourth and 3 out of 11 in the seventh, whilst all birds in the fifth and sixth passages remained free of tumours. (Fig. 1). Of the remaining 3 tumours in osteopetrosis cases one was in the progeny of affected birds (fibrosarcoma) and 2 followed embryo inoculation (one embryonal nephroma and one visceral lymphomatosis).

Fig. 1.



The distribution of neoplasia in 100 cases of osteopetrosis in relation to passages and methods of experimental infection.

It is difficult to decide if any particular source of inoculum was more frequently associated with tumour development because the numbers in the different groups are small and in 74 per cent of affected birds whole blood was the inoculum used. It is however possible that the use of filtered plasma in the third and fourth passages might have been concerned in a reduction in tumour incidence, for in three subsequent passages, in which whole blood was used for transmission, only 4 out of 38 affected birds developed neoplasia.

In birds dying with osteopetrosis (Table 4) hepatic changes other than neoplasms were observed in seven carcases. One bird died at 18 weeks with Histomoniasis. Hepatic focal necrosis was found in two (at 16 weeks and 96 weeks), hepatic cirrhosis in two (15 weeks

and 72 weeks) and two showed hepatic necrosis with extensive reactive cirrhosis at 22 and 52 weeks of age respectively.

A common finding in uncomplicated cases of osteopetrosis was a small contracted spleen which on section usually showed atrophic changes and often considerable deposits of haemosiderin. This was noted in 48 cases. The spleen was normal in size in 52 and enlarged in 8 birds which were affected with erythro-or visceral leucosis.

TABLE 4

SUMMARY OF P.M. FINDINGS IN BIRDS DYING WITH OSTEOPETROSIS

Age (wks.)	*No. of birds*	*Osteo only*	*Osteo + neoplasia*	*Osteo + Other lesions*
5–10	5	5	—	—
11–15	15	12	2	1
16–20	11	7	2	2
21–25	11	7	3	1
26–30	8	5	3	—
31–35	8	6	2	—
36–40	6	2	4	—
46–97	16	9	4	3
Total	80	53	20	7
		66·2%	27·1%	6·7%

Some affected male birds had infantile testicles and in others they were smaller than normal. This was a feature in 45 of the 83 male birds examined (54 per cent). In the remainder normal testicular development had occurred and fertility has been recorded.

When the metatarsals were involved early in the disease growth tended to be severely reduced. If these bones did not show lesions at an early age, growth was generally normal, and secondary sex characters developed. Where bone changes were very extensive, particularly involving the endosteum of the long bones with reduction in the marrow cavities, a degree of anaemia due to interference with erythropoeisis might be expected and this was noted towards the termination of the disease particularly in those birds which were stunted.

DISCUSSION

The observations recorded in Table 1 are of particular interest. It will be noted that 73·1 per cent of the 108 birds with osteopetrosis showed only bone lesions and no gross or microscopic changes in the

parenchymatous organs, whilst 20·4 per cent had osteopetrosis plus neoplasia and 6·5 per cent showed other lesions, notably hepatic necrosis and cirrhosis. Considered in relation to age groups a lower incidence of tumours might be expected in birds dying under 15 weeks, before they reached the "tumour age", and if some of these birds had lived longer they might have developed neoplasia. In the 5 to 20 weeks age group 83·7 per cent of the birds had only osteopetrosis and only 9·3 per cent showed evidence of tumours. The highest rate of tumours, 30·4 per cent, appeared in the 21 to 40 week age range. Nevertheless, even in this group 67·4 per cent of birds showed only bone lesions. In the oldest age group (40 to 100 weeks) comprising 19 birds, 63·1 per cent showed no visceral lesions but there was a much higher incidence of other lesions (15·9 per cent) notably in the liver.

It is interesting to speculate as to the cause of death in birds with osteopetrosis. The age at death was very variable from 5 weeks to 97 weeks with a fairly even distribution over this period. Numbers are small in the various age groups so the significance of percentage figures is doubtful (Table 4), but the highest level of neoplasia occurred after 20 weeks of age, whilst birds dying under 10 weeks old had no tumours. Sixty-six per cent of birds which died showed only bone lesions. Some of these birds were stunted and anaemia was often a terminal feature from reduction in haemopoietic tissue as a result of new bone formation from the endosteum of the long bones. The finding of splenic atrophy in a high proportion of birds and in some cases hepatic lesions was also reported by Gross *et al.* (1959) in birds with osteopetrosis.

Although there is a higher incidence of neoplasia in birds with osteopetrosis as compared with controls, these observations differ from those of Jungherr (1959) who stated that rapidly developing cases usually show changes typical of visceral lymphomatosis, whilst old chronic cases may show microscopic changes of a similar type. Of the 108 cases examined only 7 showed visceral lymphomatosis and 3 birds had lymphocytomata (Table 3): no gross or microscopic changes were found in the viscera in 79 birds with bone lesions.

The examination of controls and injected birds which failed to develop skeletal changes (Table 2) show a percentage of neoplasia of 1·7 and 4·7 respectively as against 20·4 per cent in birds with bone lesions. Thus there is a definite increase in the incidence of tumours in birds with osteopetrosis. The tumours observed (Table 3) were all of mesenchymal origin although there was a wide variety of types: no particular neoplasm predominated. It would seem as though the agent which induces osteopetrosis also has some influence in increasing the incidence of neoplasia, although more than two thirds of the osteopetrosis cases in this study showed only bone changes.

No proof has yet been provided that all the forms of avian neoplasia are due to infectious agents. The widespread and symmetrical distribution of the skeletal changes in avian osteopetrosis

and the clinical and radiological features, in which the primary changes are constructive and not destructive in type, suggest that osteopetrosis should not be regarded as a neoplastic disease of bone. Etiologically it would appear to resemble some forms of avian leucosis and some well recognised avian tumours (e.g. Rous sarcoma) in being transmissible through bacteria-free filtrates. It might be that those birds which develop osteopetrosis are also those which are more susceptible to tumour formation. It could be that they are carrying "tumour virus" and that the agent of osteopetrosis acts as a trigger mechanism. In support of this contention it should be noted that two birds out of 119 controls developed neoplasia. Burmester (1957) advanced the hypothesis that the visceral lymphomatosis virus mutates and causes the development of other neoplasms and Burmester, Walter, Gross and Fontes (1959) discuss the possibility that a multipotent type of virus might be involved in the leucosis complex. This envisages a virus similar to the polyoma virus described in mouse leucaemia by Mill Hill workers (Negroni, Dourmashkin and Chesterman, 1959). Alternatively a mixture of viruses might be concerned. On the other hand, the observation by Gross *et al.* (1959) that osteopetrosis is only associated with certain tumour strains favours the idea that it is caused by a separate specific virus.

If a mixture of viruses is involved a higher incidence of tumours and leucosis might be expected in those birds which were injected but failed to develop osteopetrosis. Although the incidence of neoplasia in these birds was greater than in the controls, the increase was only slight and was very low compared with birds with bone lesions (Table 1 and 2). Variation in the dose of virus received by individual birds could be one explanation for the low incidence of neoplasia in injected chicks remaining skeletally normal; in fact this could explain the failure of those birds to develop osteopetrosis. Equally well such a result could be produced by a multipotent virus, the low incidence of neoplasia in injected birds remaining skeletally normal being due again to a varying dose of virus given to individual birds; or genetic or acquired resistance may be concerned.

Of the 22 birds with neoplasia out of 108 osteopetrosis cases, only 10 had lesions which could strictly be included in the leucosis complex (7 visceral lymphomatosis and 3 erythroleucosis) that is 9·2 per cent of all the birds examined with osteopetrosis. If lymphocytoma are included the percentage is 12·0. The observations recorded here do not solve the question as to whether a multipotent virus or a mixture of viruses is involved in avian leucosis, but the low incidence of the various manifestations of the avian leucosis complex in birds with osteopetrosis, plus the presence of leucosis (although in very low incidence) in control birds, strongly favours the conception that osteopetrosis is caused by a separate virus from the other forms of avian leucosis. The fact that 73 per cent of birds with osteopetrosis showed no soft tissue changes is further evidence that this disease should be considered a separate entity from the leucosis complex and

avian neoplasia, although there is some indication that birds with osteopetrosis may be more likely to also develop soft tissue tumours than normal birds. This may be merely an index of susceptibility to this type of infective agent or possibly that infection with one virus may unmask others in susceptible recipients. In the writer's opinion these observations support the contention of Campbell (1954) that osteopetrosis should be removed from the leucotic complex in avian pathology and set apart as a distinct pathological entity.

CONCLUSIONS

Observations on the changes in the parenchymatous organs of 108 birds with avian osteopetrosis are recorded and the results are compared with normal birds and birds which were inoculated but failed to develop osteopetrosis. The possible association between osteopetrosis and the avian leucosis complex is considered. The findings tend to support the view that avian osteopetrosis is caused by a separate infective agent and the nature of the bone lesions and the low incidence of soft tissue changes typical of leucosis in affected birds provide strong evidence that the disease should be considered as a separate entity in avian pathology.

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A STUDY OF ASCORBIC ACID IN NORMAL AND KETOTIC COWS

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INTRODUCTION

It has been demonstrated in ascorbic acid deficient laboratory animals that the liver glycogen is significantly lowered (Murray and Morgan, 1946; Banerjee and Ghosh, 1947), and that glycogenesis and gluconeogenesis are disturbed or diminished (Bacchus and Heiffer, 1954; Murray and Morgan, 1946, 1948; McKee, Cobbey and Geiman, 1949). It has also been demonstrated in the rat which, like the ruminant, synthesises its own ascorbic acid, that glucose is utilised for this synthesis (Horowitz, Doerschuk and King, 1952; Burns and Mosbach, 1956). Meiklejohn (1953) in his review of literature on ascorbic acid states—"the greatest interest now centres on 'stress' situations. Under some conditions known to deplete ascorbic acid in the adrenal cortex, there is also evidence of increased catabolism of the vitamin in man."

In view of the concept of Shaw, Hatziolos and Leffel (1950) which was based on Selye's theory, that the deficiency of carbohydrate in ketosis was due to the stresses of parturition and lactation leading to a temporary exhaustion of the pituitary causing a relative insufficiency of the adrenal corticosteroids, it is interesting to note the possible relationship between ascorbic acid, stress conditions and carbohydrate metabolism.

Saarinen and Shaw (1950) have estimated plasma, liver and adrenal ascorbic acid levels in cows with ketosis and adrenal ascorbic acid levels in fasting cows. In the cows with ketosis they found a marked variation in the plasma levels and low values for adrenal and liver content, while those in the fasting cows were the lowest levels observed. Puntriano (1951) recommended ascorbic acid along with adreno-corticotrophic hormone in the treatment of ketosis without quoting any experimental evidence and gave as a reason that it factilitated the building-up of corticosteroid hormone in the adrenal cortex. Bizet (1957) has also reported the successful use of massive daily doses of Vitamin C in the treatment of resistant paraplegia due to hypocalcaemia or acetonaemia.

The following investigation into the possibility of ascorbic acid being involved in the condition of ketosis was carried out as a result of these observations.

METHODS

Ascorbic acid. Total ascorbic acid content estimated by using the colorimetric method of Roe and Kuether (1943) as modified by Schaffert and Kingsley (1955).

Glucose. Determined by the method of Somogyi (1945) using his modified copper reagent (1952) and the colour-producing arsenomolybdate reagent of Nelson (1944).

Total acetone bodies and free acetone. Thin and Robertson's method (1952)

Eosinophils. Counted in a Fuchs Rosenthal chamber using the diluting fluid of Randolph and Arbor's fluid (1944) as modified by Pilot (1950).

Ascorbic acid saturation tests. A slightly modified version of the urine-collecting apparatus described by Gorski, Blosser, Murdock, Hodgson, Soni and Erb (1957) was used. Urine was collected at eight-hourly intervals, the volume measured and aliquots taken for estimation of ascorbic acid content. After a 24 hour control period a measured quantity of ascorbic acid was injected subcutaneously at the 24th and then again at the 48th hour. Output of ascorbic acid over subsequent 24 hour periods was calculated. The necessity for controlled feeding during this test was dismissed in view of the work of Vavich, Dutcher, Guerrant and Bechdel (1945) and Wallis (1943) who have shown in cows that any increase or decrease in the dietary intake of ascorbic acid has little effect on the levels of urinary excretion. This was convenient because the diet of all the cows with ketosis was disturbed anyway.

Experimental procedure. The whole blood levels for ascorbic acid, glucose, total acetone bodies and free acetone were studied at weekly intervals during the pre- and post-parturient period in nineteen normal dairy cows from February to June 1958. Besides obtaining normal values, it was hoped that any trend or relationship between these levels could be witnessed. It was also hoped that if any animal developed ketosis during the time of study a deviation from the normal might be observed. One of the nineteen cows (i.e. Cow 1) developed ketosis and the results obtained for this animal are described.

In addition to these animals several cases of ketosis were also studied. As a means of observing the body saturation and excretion of ascorbic acid in cases of ketosis, saturation tests were conducted over a period of 72 hours in Cows 1 to 4. The observations made were compared with similar tests on normal post-parturient cows (i.e. Cows D.11, D.19 and E.7). The test was also repeated on Cow 2 one month after she had recovered from ketosis. At the same time as the saturation test, eosinophil counts and biochemical estimations of ascorbic acid, glucose and ketones were carried out on the blood of these clinical cases before and after injection of ascorbic acid. Milk yields were also recorded. If the response to ascorbic acid was unsatisfactory then a more conventional type of treatment was adopted.

RESULTS

Blood Constituent Levels in Normal Dairy Cows during the Prepartum and Postpartum Period

The levels are summarised in Table 1. Values for ascorbic acid ranged from 0·08 to 0·62 mg./100 ml. Levels appeared to fall slightly just before parturition or during the week of parturition, and were more consistently high during the four to eight week period of lactation.

Glucose values ranged from 22 to 83·5 mg./100 ml. All the cows showed an increase towards parturition, the highest values being obtained on the day of parturition. In nine cows the levels dropped below 30 mg./100 ml. at some stage during the first five weeks of lactation. After five weeks this slight hypoglycaemia only occurred

TABLE I

WHOLE BLOOD LEVELS OF CONSTITUENTS IN NORMAL PRE- AND POST-PARTURIENT DAIRY COWS

		Weeks Prepartum						*Week of Calving*	*Weeks Postpartum*							
		6	*5*	*4*	*3*	*2*	*1*	*0*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*
No. of cows sampled		*6*	*5*	*10*	*11*	*12*	*16*	*17*	*16*	*13*	*17*	*16*	*17*	*14*	*17*	*13*
Ascorbic acid (mg./100 ml.)	Mean and Standard Error	0·32 (±0·034)	0·32 (±0·051)	0·32 (±0·036)	0·39 (±0·036)	0·30 (±0·021)	0·29 (±0·023)	0·30 (±0·021)	0·39 (±0·029)	0·37 (±0·018)	0·38 (±0·027)	0·39 (±0·031)	0·40 (±0·027)	0·46 (±0·014)	0·39 (±0·026)	0·44 (±0·024)
	Range	0·18–0·40	0·22–0·53	0·08–0·43	0·15–0·58	0·18–0·42	0·15–0·45	0·19–0·52	0·15–0·61	0·30–0·55	0·15–0·58	0·19–0·62	0·20–0·61	0·37–0·53	0·15–0·62	0·31–0·62
No. of cows sampled		*6*	*5*	*10*	*11*	*10*	*17*	*16*	*16*	*15*	*17*	*17*	*17*	*17*	*17*	*13*
Glucose (mg./100 ml.)	Mean and Standard Error	36·4 (±2·59)	33·8 (±2·90)	34·3 (±1·31)	36·2 (±1·19)	38·0 (±1·94)	41·6 (±1·36)	48·0 (±2·23)	37·6 (±1·28)	34·0 (±1·50)	38·0 (±1·78)	36·5 (±1·92)	38·5 (±1·71)	36·3 (±1·44)	40·0 (±1·77)	40·9 (±2·20)
	Range	28·6–45·7	22–39·5	25·8–39	29·2–42	27·7–45·7	31·5–52	36–83·5	30·7–51	25·8–49·7	23·3–51·2	23·5–52	28·4–51·2	26·2–44·7	24·7–49·2	26·2–58·7
No. of cows sampled		*6*	*5*	*10*	*11*	*11*	*17*	*17*	*16*	*16*	*17*	*18*	*17*	*17*	*17*	*13*
Total ketones (mg./100 ml.)	Mean and Standard Error	3·0 (±0·76)	5·2 (±0·82)	4·1 (±0·76)	2·7 (±0·66)	2·5 (±0·64)	3·4 (±0·73)	3·3 (±0·59)	2·6 (±0·44)	4·5 (±1·03)	4·7 (±0·53)	5·3 (±0·78)	4·6 (±0.66)	3·9 (±0·53)	4·0 (±0·76)	2·7 (±0·43)
	Range	0·52–5·9	2·35–7·7	0·65–7·6	0–7·21	0·52–6·6	0–8·43	0–8·1	0–6·6	0·52–20·5	1·13–9·65	0–12·08	1·13–12·08	1·13–8·43	1·13–14·5	1·13–7·21
No. of cows sampled		*6*	*5*	*10*	*11*	*11*	*17*	*17*	*16*	*16*	*17*	*18*	*17*	*17*	*17*	*13*
Free acetone (mg./100 ml.)	Mean and Standard Error	0·98 (±0·59)	1·49 (±0·33)	1·92 (±0·64)	0·94 (±0·29)	0·6 (±0·44)	1·55 (±0·46)	1·35 (±0·36)	0·93 (±0·26)	1·56 (±0·36)	1·77 (±0·49)	1·47 (±0·36)	1·67 (±0·34)	1·49 (±0·36)	1·5 (±0·39)	0·56 (±0·19)
	Range	0–3·56	0·52–2·35	0–6·2	0–2·35	0–5·0	0–6·0	0–5·0	0–2·91	0–5·39	0–5·99	0–5·99	0–4·75	0–4·17	0–5·99	0–1·74

TABLE 2

MEAN VALUES AND ANALYSIS OF VARIANCE ON BLOOD LEVELS OF CONSTITUENTS

Eight normal pre- and post-parturient dairy cows

Blood Constituents	*Weeks*							*Standard error of mean*	*Significance*
	Prepartum			*Calving*	*Postpartum*				
	3	*2*	*1*	*0*	*1*	*2*	*3*		
Ascorbic acid (mg./100 ml.)	·385	·324	·286	·292	·387	·371	·380	(± ·033)	Not significant ($N_1 = 6$; $N_2 = 42$; $F = 1·86$)
Glucose (mg./100 ml.)	36·9	37·0	40·7	49·5	38·1	33·7	36·7	(± 2·78)	Significant at 1% ($N_1 = 6$; $N_2 = 42$; $F = 3·45$)
Total acetone bodies (mg./100 ml.)	3·24	1·94	2·71	1·98	2·51	5·01	5·61	(± ·717)	Significant at 1% ($N_1 = 6$; $N_2 = 42$; $F = 5·78$)
Free acetone (mg./100 ml.)	1·09	0·21	1·03	0·63	0·41	1·90	1·87	(± ·524)	Not significant ($N_1 = 6$; $N_2 = 42$; $F = 1·63$)

TABLE 3

ASCORBIC ACID SATURATION TEST ON NORMAL POST-PARTURIENT AND KETOTIC COWS

Volume of Urine, Concentration of Ascorbic Acid and Total Ascorbic Acid Excreted at Eight Hourly Intervals

Animals	*Ascorbic acid*	*Time of collection Hours*	*Volume of urine collected Litres*	*Ascorbic acid concentration Mg./litre*	*Total excretion of Ascorbic acid Mg*
Normal post-parturient cows					
(i) D.19— Control	No Ascorbic acid injected	8	2·2	27·5	60·5
		16	2·3	22·25	51·17
		24	3·05	22·0	67·1
		32	2·35	24·75	58·16
		40	2·3	38·5	88·55
		48	2·08	34·25	71·24
		56	1·3	40·75	52·97
		64	2·42	36·25	87·72
		72	2·42	40·75	98·61
(ii) D.11	Injected IG at 48 hours	8	1·85	25·38	46·95
		16	2·9	21·5	62·35
		24	2·17	19·5	42·31
		32	3·1	28·0	86·8
		40	1·67	46·95	78·46
		48	1·71	36·0	61·56
		56	1·85	56·0	103·6
		64	1·65	54·0	89·1
		72	2·85	39·5	93·08
(iii) E.7	Injected 3G at 24 and 48 hours	8	2·5	35·0	87·5
		16	1·98	27·0	53·46
		24	1·88	36·0	67·68
		32	2·1	340·0	714·0
		40	1·39	137·5	191·12
		48	1·6	92·0	147·2
		56	1·89	375·0	708·75
		64	3·06	187·5	573·75
		72	1·91	45·25	80·69
2. *Cows with ketosis*					
Cow 1	Injected IG at 24 and 48 hours	8	9·26	43·5	402·81
		16	7·1	44·5	315·95
		24	8·81	36·0	317·16
		32	8·8	15·5	136·4
		40	7·11	11·5	81·77
		48	11·35	7·5	85·13
		56	—	21·0	—
		64	—	28·0	—
		72	—	14·25	—

Table 3—*Continued*

Animals	*Ascorbic acid*	*Time of collection Hours*	*Volume of urine collected Litres*	*Asorbic acid concentration Mg./litre*	*Total excretion of Ascorbic acid Mg*
Cow 2	Injected IG at 24 hours and 2G at 48 hours	8	3·87	60·0	232·2
		16	3·71	33·0	122·43
		24	2·34	53·5	125·19
		32	1·79	87·5	156·62
		40	2·93	60·64	177·67
		48	1·78	51·0	91·63
		56	2·97	155·0	460·35
		64	3·16	110·0	347·6
		72	3·05	80·0	244·0
Cow 3	Injected 3G at 24 and 48 hours	8	8·65	22·06	190·81
		16	7·85	24·52	192·48
		24	3·51	27·75	97·40
		32	1·91	74·88	143·02
		40	4·22	18·23	76·93
		48	2·2	49·75	109·45
		56	2·37	110·0	260·7
		64	3·19	35·95	114·68
		72	1·21	36·0	43·56
Cow 4	Injected 4G at 24 hours	8	4·35	92·5	402·83
		16	3·52	66·5	234·08
		24	3·95	57·0	225·15
		32	3·57	420·0	1499·4
		40	2·6	305·0	793·0
		48	2·28	261·0	595·08
		56	—	—	—
		64	—	—	—
		72	—	—	—

in four cows. A similar drop in levels was noted in five out of thirteen cows during the two to six weeks prepartum period.

The total ketones ranged from 0 to 20·5 mg./100 ml. and free acetone from 0 to 6 mg./100 ml. During the postpartum period eight cows showed a tendency for the total ketone levels to rise; this was not accompanied by a rise in free acetone.

As the weekly sampling was not complete for every cow in the above study, statistical analyses were carried out on eight cows in the groups for which full records were available for the seven week period beginning three weeks prepartum and ending three weeks postpartum. The mean values and results of an analysis of variance on these records are shown in Table 2. There was no significant trend in blood ascorbic acid levels throughout the period studied. Glucose levels showed a significant increase during the week of parturition. Levels for total ketones rose during the second and third

week postpartum, and the rise during the third week was significant. There was no apparent trend in the free acetone levels. No relationship could be found to exist between the ascorbic acid and the glucose levels.

Comparison of Ascorbic Acid Saturation Tests in Normal Post-parturient Cows and Clinical Cases of Ketosis

Values for the eight-hourly output of urine, concentration of ascorbic acid and total ascorbic acid excreted by normal post-parturient and ketotic cows are summarised in Table 3.

The excretion of ascorbic acid by the ketotic cows during the initial 24 hour period was high. Numbers 1 and 3 however appeared to show a retention of ascorbic acid after injection. In Cow 1 this was exhibited by a marked reduction in the urinary concentration and total output of ascorbic acid during the 24 hours after injection. It will also be observed in Figure 2 that this animal showed a distinct drop in blood ascorbic acid levels during the first 16 hours after injection and only began to rise slightly at 24 hours. The effect on the urinary levels seen in this cow was the opposite to that seen in the normal cow D.11, which was injected with a similar amount of ascorbic acid at the end of a 48 hour control period. However, the slight response shown by the normal animal could not be regarded as significant. A further injection of ascorbic acid at the end of 48 hours in Cow 1 raised the urinary concentration, but this was still 20 mg./litre less over 24 hours than observed during the control period. Unfortunately total excretion could not be calculated during this period because of a fault in the collecting apparatus. Cow 3 also appeared to retain an abnormal amount of the ascorbic acid injected, that is, if the values obtained for this animal are compared with those shown in a comparable experiment with the normal cow No. E.7.

The other ketotic Cows, 2 and 4, although excreting like 1 and 3 comparable amounts of ascorbic acid during the control period, did not show any retention of the vitamin after it was injected. In Cow 4 the injection of a relatively high dose of ascorbic acid resulted in a marked increase in the urinary concentration and total output. Due to the positive response shown by this animal further sampling was not warranted.

An apparent increase in the initial excretion of ascorbic acid in the urine of cows with ketosis raised several problems, and it had to be established whether it was normal for a particular cow, or a manifestation of ketosis. Unfortunately only one animal Cow 2, was available for repetition of the experiment. One month after this animal had recovered from ketosis, she was subjected to exactly the same experiment using the same quantities of ascorbic acid as before. The results showing the eight-hourly levels are given in Table 4. If these results are compared with those shown in Table 3 it will be observed that during the first 48 hours the picture was similar, but on repeating the injection of ascorbic acid at the end of 48 hours

there was, unlike the previous experiment, only a slight response in the excretion rate and total output.

Despite the inconclusive results of this experiment and the obvious need for further investigational work, the most significant clinical feature observed during this study was the presence of polyuria in two cases. As an early diagnosis was made in these cases it may be that polyuria is essentially a symptom of the early stages of the condition. These animals were passing excessive volumes of pale, watery urine of low S.G. In Cow 1 the total volume of urine passed during the first 48 hours amounted to 52.42 litres. It was also noted in Cow 3 that when, after the first 24 hours this animal showed a noticeable clinical improvement, the amount of urine being excreted was considerably reduced and the S.G. was raised. Neither animal showed evidence of nephritis. Whether polydipsia is also an accompanying clinical feature has yet to be determined.

TABLE 4

ASCORBIC ACID SATURATION TEST—REPEAT EXPERIMENT ON COW 2

Volume of urine, concentration of ascorbic acid and total ascorbic acid excreted at eight-hourly intervals

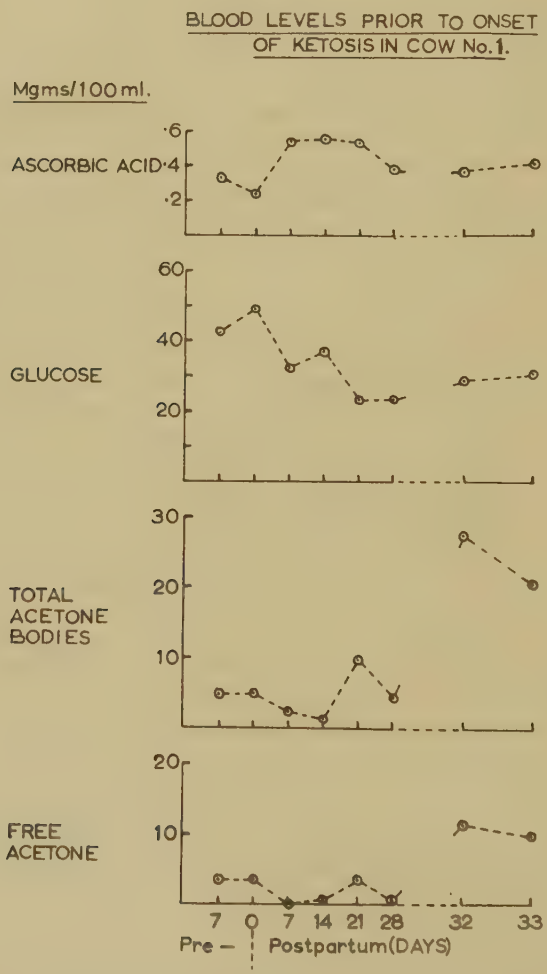
Animal	*Gm. of Ascorbic acid injected and times of injection*	*Time of collection* *Hours*	*Volume of urine* *Litres*	*Ascorbic acid* *Mg. litre*	*Total excretion of Ascorbic acid* *Mg.*
Cow 2, Sampled one month after recovering from ketosis	Injected IG at 24 hours and 2G at 48 hours	8	2·78	59·0	164·02
		16	2·8	53·25	149·36
		24	2·95	35·75	105·46
		32	2·79	58·0	161·82
		40	2·0	54·0	108·0
		48	1·84	54·0	99·36
		56	2·4	103·0	247·2
		64	1·71	60·0	102·6
		72	3·89	31·5	122·53

Blood Constituent Levels in Ketotic Cows and the Effects of Injecting Ascorbic Acid

It can be seen from Fig. 1 that Cow 1 which had been blood sampled at weekly intervals with the normal cows showed no drop in ascorbic acid levels between the last sample taken on the 28th day postpartum when she was still normal and the one taken on the 32nd day when signs of ketosis were present. In fact, the lowest blood ascorbic acid level seen was during the week of calving. The blood glucose levels in this cow dropped after parturition, then rose slightly at the second week and then remained at a low level from the third week onwards. There was also a slight increase in total

ketone bodies during the third week, but they dropped again at the fourth week. The glucose level, however, was lower on the 28th day than that observed on the 32nd day and in the space of four days the total ketones increased rapidly from 4.17 mg. to 27.41 mg./100 ml.

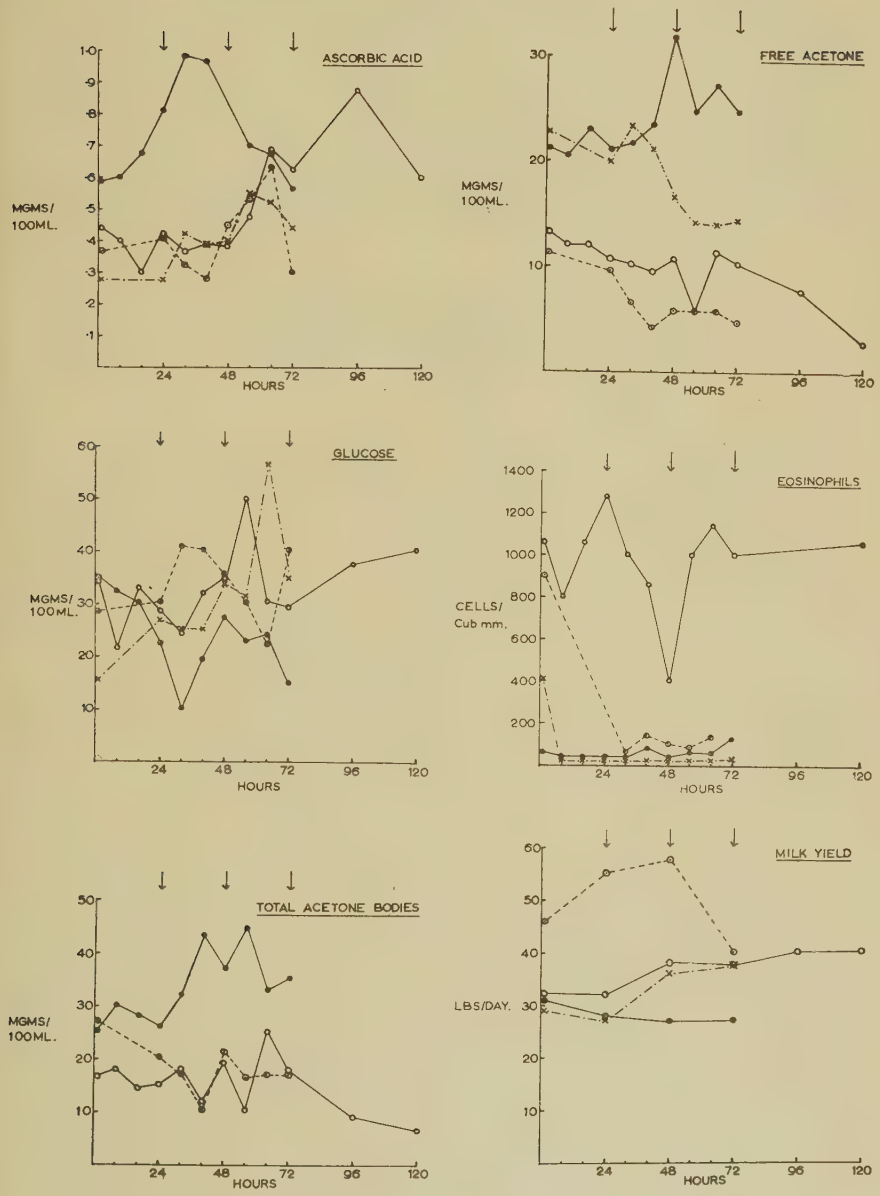
Fig. 1



In Figure 2 the effect of ascorbic acid on the blood levels and milk yields are compared for the four cows with ketosis. The initial blood samples from all these cases showed normal values for ascorbic acid. Cow 2 recovered completely with repeated injections of ascorbic acid, but how much of this recovery was spontaneous could not be assessed. However, in this animal and in Cows 1 and 3 injection of ascorbic acid appeared to raise the blood glucose level sometimes after an initial fall, and also reduced the free acetone

Fig. 2

BLOOD CONSTITUENT LEVELS AND MILK YIELDS OF KETOTIC COWS BEFORE AND AFTER INJECTION OF ASCORBIC ACID



Key:—

Cow No.1 — 1G. Ascorbic Acid injected at 24&48 hours.

" " 2 — " " " " " 24hours and 2G. at 48 & 72 hours.

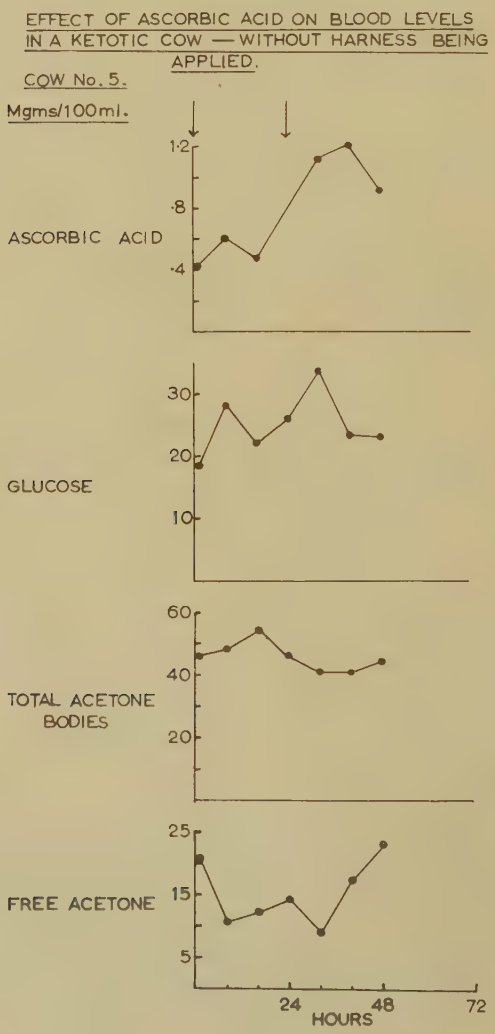
" " 3 — 3G. Ascorbic Acid injected at 24&48 hours.

" " 4 — 4G. " " " " 24hours and 1G. at 48 hours.

Times of injection.

levels. There was also an improvement in appetite in these animals and their milk yield either increased or was maintained, apart from Cow 1 which suddenly dropped in milk yield at the 48th hour. The initial progress shown after ascorbic acid injections was not maintained in Cows 1 and 3, and other treatment was adopted before recovery was complete. In these animals which showed a favourable response initially the blood eosinophil levels were moderately raised, whereas in Cow 4 which did not show any satisfactory response to ascorbic acid, the levels were very low. It is also of great interest to observe the marked reduction in circulating eosinophils in Cows 1, 2 and 3 during the period prior to injection of ascorbic acid, when

Fig. 3



the only interference was the stress of wearing a strange harness and apparatus. How much of the subsequent response observed in these animals was due to the application and presence of the urine collecting apparatus is debatable. The effect on glucose and free acetone levels after injection with ascorbic acid was more marked within eight hours than that observed over the first 24 hours when the animals were only wearing the apparatus and had no treatment. During this control period, however, there was a rise in glucose in Cows 1 and 3 and a drop in free acetone levels in 1, 2 and 3, and the milk yield of 1 rose significantly.

It is shown in Figure 3 that in the case of Cow 5 in which no apparatus was fitted, there was an obvious reduction in free acetone levels and a slight but transient increase in glucose levels after injection with ascorbic acid. Again, the apparent improvement in this animal was not maintained and conventional treatment had to be adopted to complete the recovery.

DISCUSSION

In the study of normal cows the fluctuation in ascorbic acid levels could not be correlated with the glucose levels in the blood. Although there appeared to be a drop in levels towards parturition when the glucose levels increased, this drop was not statistically significant. The consistently high levels observed during the four to eight week postpartum period could be associated with the onset of oestrus as observed by Phillips, Lardy, Boyer and Werner (1941). In Cow 1 which developed ketosis there was no appreciable change in the blood ascorbic acid levels prior to the onset of the condition and all the other cases of ketosis examined had normal blood values. This observation agrees with the work of Saarinen and Shaw (1950) that there is no direct relationship between the blood levels of ascorbic acid and the condition of ketosis.

The significant increase in blood glucose levels at parturition and the apparent tendency for some animals to drop in levels during the first few weeks of lactation has been observed by other workers (Godden and Allcroft, 1932; Shaw, 1943; Van Soest and Blosser, 1954; Horrocks and Paterson, 1957, 1960). Shaw found that the decrease in blood glucose after parturition continued for another few days before symptoms of ketosis were observed. Cow 1 showed this customary drop after parturition, but unlike the cases quoted by Shaw there was a transient rise during the 2nd week prior to a drop during the 3rd and 4th weeks, when she was behaving normally, and then a slight rise again on the day she was examined and found to have ketosis. The rise in ketone bodies in this animal, although slight during the 3rd week, did not increase gradually as in the cases quoted by Shaw, but suddenly increased from a normal level to a clinical one between the 28th and 32nd days postpartum.

In the normal cows studied, over 60 per cent. showed a hypoglycaemia at some stage during the first 8 weeks of lactation. This

fact compares well with the observation of Horrocks and Paterson (1957). No reference can be found to compare with the observation in this study, that several normal cows showed a tendency to drop in glucose levels 2 to 6 weeks prior to parturition.

The rise in ketone bodies after parturition in normal cows has been recorded by Boddie (1935) who demonstrated that although the increase was not significant it was mainly due to an increase in *B*-hydroxybutric acid levels. This observation is borne out by the levels of total acetone bodies demonstrated in this study which showed a significant rise during the third week after parturition but were not accompanied by a significant rise in free acetone levels. The recent work by Horrocks and Paterson (1960) confirms this observation.

The results displayed by Cow 2 in the repeat ascorbic acid saturation experiment seemed to indicate that the apparent increase in urinary excretion and retention of ascorbic acid witnessed in the ketotic cows was not significant. The value of such a test in the bovine is doubtful and further investigational work is required. This still does not explain the very marked rapid decrease in the urinary concentration of ascorbic acid after injection of the vitamin as observed in Cow 1, unless there may have been a true tissue depletion of ascorbic acid in this animal. The particular batch of ascorbic acid used for injection in this animal was examined for potency and found to be sound.

The clinical observation of polyuria existing in cases of ketosis is believed to be an original one. It has been reported by Van Soest, Blosser, Ward, Crilly and Adam (1952) that in three cows with ketosis they found the average 24 hour excretion of urine was 9·5 litres. This is considerably less than that observed in the cases described in this paper. The figures of Fuller (1928) for the normal range of urine excretion in dairy cows are 8·8 to 22·6 kg. with an average of 14·4 kg. but it has been shown by Ellet and Holdaway (1917, 1920) who also quote Lusk (1906) that the amount of urine excreted by dairy cows is influenced by the excess protein ingested in the diet. They also stated that cows receiving a basic diet rich in protein will excrete four times as much urine as a cow receiving the same diet but containing less than half this quantity of protein. With this fact in mind it is interesting to compare the initial 24 hour volume of urine excreted by the four ketotic cows with the normal cows and in the repeat experiment on Cow 2 remembering that the former were refusing to eat concentrates whereas the normal cows were consuming up to 14 lb. of concentrates per day. This observation could account for the supposition hitherto held that symptoms of rapid loss in bodyweight and dehydration were the result of inanition or increased catabolism of body fat and protein alone. Could it be that the animal loses weight rapidly in the first instance through an excessive water loss from the body? Also, could it be that the polyuria is the result of a temporary insufficiency of the

pituitary and decreased secretion of the antidiuretic hormone, or a deficiency of the adrenocortical hormones causing a water loss and disturbance in the electrolyte balance? It is interesting to note in this connection that the sudden drop in eosinophil levels in Cow 3 also accompanied a rapid reduction in urinary excretion. These observations have demonstrated the necessity for a more thorough and extensive clinical approach to the condition and a combination of clinical observations and biochemical findings for possible classification of the stages or types of ketosis encountered in the field.

As the fundamental role of ascorbic acid in metabolism is unknown, it is uncertain what mechanism is involved in the reduction of free acetone and fluctuation in glucose levels as observed in four out of five cases of ketosis after injection of ascorbic acid. Although the urine collecting apparatus had a similar influence on the level of these blood constituents during the control period the effect was not so marked, and it was also established in the one case which had no harness fitted that ascorbic acid alone could be responsible for these effects. It has been shown by Takeda and Hara (1955) and Hara (1957) that ascorbic acid may possibly play some role in the tricarboxylic acid cycle and provide essential links in catabolic enzyme reactions. It is also interesting to note that Sealock and Silberstein (1940) demonstrated the incomplete breakdown of the ketogenic amino-acid tyrosine in guinea pigs with scurvy.

Some of the observations on eosinophil levels in this paper are of interest in view of the work by Shaw (1955) who claimed that the injection of adrenaline had no effect on the circulating eosinophil levels in ketotic cows, but that it had in normal cows, and that this was an indication of temporary exhaustion of the anterior pituitary in ketosis. It has since been shown by Alexander (1958) that injection of adrenaline has no effect on circulating eosinophil levels in the normal bovine. Since the cows with ketosis described in this paper showed a very marked reduction in eosinophil levels with the application of the harness alone, this would seem to indicate that in fact these animals were capable of responding to a new 'stress'. The stimulation of the pituitary/adrenal axis is further indicated by the rise in blood glucose and drop in ketone levels during this period. Apparent insufficiency of the anterior pituitary may therefore only be an incidental factor in the condition of ketosis, and may only be a reflection on the stage of the condition.

In conclusion, it is apparent that ascorbic acid has no primary role in spontaneous ketosis, but the observations made in this study suggest that it may play some secondary role. For this reason it may have some place as an adjuvant when combined with a more conventional drug in the treatment of the condition.

CONCLUSIONS

Weekly estimations of whole blood ascorbic acid, glucose and

ketones were made on nineteen normal dairy cows during the prepartum and postpartum periods.

Ascorbic acid levels showed no significant trend or relationship to glucose. The glucose levels increased significantly at parturition and in 60 per cent. of cows they dropped below 30mg./100 ml. at some stage during the first eight weeks of lactation. Total acetone bodies increased significantly at three weeks postpartum but there was no change in free acetone.

Ascorbic acid saturation tests were carried out in ketotic and normal lactating cows. Two cases of ketosis appeared to show abnormal retention after injection of test doses of ascorbic acid, but observations suggest that this test may not be reliable in the bovine.

The observation that polyuria is a clinical feature in the early stages of ketosis is discussed.

Blood ascorbic acid values in cases of ketosis were normal. Four out of five cases of ketosis injected with ascorbic acid showed a favourable clinical response initially, but improvement was maintained in only one case.

Contrary to the theory of pituitary exhaustion certain observations indicated that ketotic cows are capable of reacting to 'stress'.

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THE IDENTIFICATION OF A HAEMOPHILUS-LIKE ORGANISM ASSOCIATED WITH PNEUMONIA AND PLEURISY IN THE PIG

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INTRODUCTION

In a previous paper, Pattison, Howell and Elliot (1957) described a Haemophilus-like organism isolated from pneumonic pig lung. Growth of the organism was poor on the media used and efforts at identification failed, although it appeared likely that the organism was a member of the genus Haemophilus and differed from *Haemophilus influenzae suis*.

Intratracheal injection of pigs with pure cultures of the organism failed to produce disease, but when swine fever virus was present in inoculated cultures a histologically characteristic pneumonia with pleurisy developed. These lesions were quite different from those produced by injecting swine fever virus alone. The pneumonia and pleurisy attributed to the injected Haemophilus-like organism were described in detail by Pattison *et al.*

Following this work an attempt was made to preserve the organism in the dried state, but when re-examined some time later the cultures were non-viable and the organism was lost.

At a later date another strain of what appeared to be the same Haemophilus-like organism was isolated in this laboratory. However, there were two important differences between this strain and the original: (a) it grew relatively well on media suited to Haemophilus organisms and of the kind used with only moderate success in the earlier work (b) in pure culture and without the addition of swine fever virus it produced pneumonia with pleurisy when inoculated intra-tracheally into pigs (Mackenzie, personal communication). The histo-pathological lesions produced in experimental pigs by this new strain were, however, identical with those found in the earlier work when the original strain was inoculated into pigs along with swine fever virus. As these lesions were histologically unusual it seemed likely that the new strain was the same organism as that originally described, but that it was culturally more tractable and experimentally more pathogenic.

The identification of a pathogenic micro-organism usually depends more on its cultural and serological characteristics than on the nature of the lesion it produces in experimental animals, but in this instance two organisms of apparently the same genus had been isolated from pneumonic and pleuritic pig lungs and each was apparently capable of producing a histologically characteristic and unusual pneumonia with pleurisy when injected intra-tracheally into experimental pigs. It appeared likely, therefore, that in spite of the cultural shyness of the one and the relatively easy culture of the other they were variants of the same organism. Further, it was considered possible that the apparent non-pathogenicity of the original strain, unless injected along with swine fever virus, might be partly due to the fact that it grew so poorly on artificial media that only

relatively few organisms were available for inoculation of experimental pigs.

It was postulated, therefore, that if a small inoculum of the new strain could be shown to be non-pathogenic, and if this small inoculum could then be made pathogenic by addition of swine fever virus, this might provide a reasonable explanation for the apparent difference in pathogenicity of the old and new strains and would indicate that they were variants of the same organism.

This paper describes the pathogenicity of the new strain of the Haemophilus-like organism and compares its cultural, serological, and pathogenic characteristics with those of *H. influenzae suis*—the other member of the genus Haemophilus most likely to be isolated from pig lungs. Reasons are given for the conclusion that this Haemophilus-like organism is a member of the species *H. para-influenzae*.

MATERIALS AND METHODS

Strains of Haemophilus. We are indebted to Dr. Margaret Pittman of the National Institute of Health, Bethesda, U.S.A., for three Shope strains of *H. influenzae suis* 3442, 3498, and 3510/14, and four human strains of *H. para-influenzae* 429, 655, 678 and 759; to Dr. K. Bakos of the Statens Veterinärmedicinska Anstalt, Stockholm, for his type strains A, B, C and D (Bakos, 1955) of *H. influenzae suis*; and to Mr. A. Mackenzie of Compton for the Haemophilus-like organism strain 5408 used in this work and for other isolations of the same organism from pigs in the same herd.

Bacteriology. For routine subculture and preparation of suspensions for serological work, the medium containing X and V growth factors described by Matthews (1960) was used. For determining growth factor requirements, assay media containing either 1:1,000 Hemin (Light & Co. Ltd.)* or 1:1,000 diphosphopyridine nucleotide (Coenzyme I: Light & Co. Ltd.) in 2 per cent. Oxoid bacteriological peptone water were used. The final growth factor concentrations in these assay media were 1:31,250 of pure factor X, or 1:312,500 of pure factor V. The 1:1,000 Hemin solution was made by dissolving 0·1 g. of Hemin in 100 ml. of 1 per cent. Na_2CO_3. For determining requirements of X + V factors, the same concentrations of X and of V were combined in 2 per cent. peptone water; this medium was also used for growing organisms for pig inoculation.

Fermentation reactions were measured in the assay medium containing X + V factors together with Andrade's indicator and 1 per cent. of the 'sugar' under test. Reactions were read after seven days at 37°C.

Viable counts were made by the method of Miles and Misra (1938).

For examination of the lungs of inoculated pigs, five cultures on to the routine medium were made from each set of lungs—from the right and left apical and diaphragmatic lobes and from a bronchial gland—by rubbing the tissue over the surface of the medium. Colonies resembling Haemophilus were picked off after overnight incubation. Positive identification of all isolations of the Haemophilus-like organism was based on specific precipitation with the homologous serum and by fermentation reactions.

*Light & Co., Ltd., Poyle Trading Estate, Colnbrook, Bucks, England.

Serology. Sera were prepared by the intravenous injection of rabbits with living Haemophilus cells of the appropriate strain grown overnight on the routine medium, washed once in physiological saline and resuspended in saline to give an opacity equal to Brown's Tube No. 4. A fresh culture was used on each occasion. Rabbits were inoculated with initial doses of 0·2 ml. on three consecutive days; a week later with 0·5 ml. on three consecutive days, and in subsequent weeks with 1·0 ml. on three consecutive days until a serum of suitable titre was obtained. Living cells were found to give better sera than formolised cells.

Absorption of sera was carried out by adding to 1·0 ml. of serum an equal volume of almost dry cells killed by heating at 100°C for 10 minutes; the cells were obtained by centrifuging the washings in physiological saline of the overnight growth from five 4″ Petri dishes.

The antigen extract for the precipitin test was prepared by the method of Fuller (1938) from centrifuged cells obtained after overnight growth on the routine medium.

The precipitin test was carried out in 3 cm. glass tubes of 2 mm. internal diameter stuck in 'plasticine' racks. About 0·1 ml. of serum was used for each test and about the same volume of extract was layered on top. The test was read after 10 minutes at room temperature.

Histology. Eleven blocks of tissue—from the apical, cardiac and diaphragmatic lobes of the right and left lungs, and from the intermediate lobe—were examined from every animal. Tissues were fixed in formol-saline, embedded in paraffin wax, and stained with haematoxylin and eosin.

Swine fever virus. The B.A.I. strain of swine fever virus, kindly supplied by the Central Veterinary Laboratory, Weybridge, was used. As received at Compton the virus was contained in defibrinated whole blood, of which 1·0 ml. of a 1:1,000 dilution in distilled water injected subcutaneously was known to produce clinical evidence of disease within three to four days and death within 14 days. Pigs inoculated intratracheally received approximately twice this dose contained in 20 ml. of the appropriate Haemophilus culture.

Experimental animals. The pigs used were about eight weeks old and were obtained from the virus-pneumonia-free Compton herd of Large Whites. Intra-tracheal inoculation was carried out under intraperitoneal Nembutal anaesthesia with the pig on its back and the head at a slightly higher level than the tail. The volume inoculated was 20 ml. in every case.

RESULTS

The Cultural Characteristics of the Haemophilus-like Organism compared with those of Human Haemophilus para-influenzae *and of* Haemophilus influenzae suis

Requirements for the growth factors X and V. In testing the growth factor requirements of members of the genus Haemophilus, preliminary observations showed that when a heavy inoculum of many strains was transferred to peptone water containing neither X nor V, viability, without multiplication, was maintained for up to 48 hours. It was necessary, therefore, to use a small standard inoculum when the growth requirements of a strain were under test. This

standard was fixed at approximately 100 viable organisms. To test the growth requirements of a strain the standard inoculum contained in 0.02 ml. was subinoculated into 5·0 ml. of each of the assay media. The occurrence of growth was determined turbimetrically after overnight incubation at 37°C. The growth requirements of four strains of the Haemophilus-like organism, three strains of human *H. para-influenzae*, three Shope strains and four Bakos strains of *H. influenzae suis* are given in Table 1. The number of plus signs in

TABLE 1

REQUIREMENTS FOR GROWTH FACTORS X AND V

Four strains of the Haemophilus-like organism, three human strains of *Haemophilus para-influenzae*, and three Shope strains and four Bakos strains of *Haemophilus influenzae suis*.

Organism tested	*No. of strains*	*Requirements for growth factors*		
		X only	*V only*	*X + V*
Haemophilus-like organism	4	0	+++	++
Human *Haemophilus para-influenzae*	3	0	+++	+++
Shope *Haemophilus influenzae suis*	3	0	+	+
Bakos *Haemophilus influenzae suis*	4	0	++	++

0 = No growth
+ to +++ = Growth in increasing density

the table indicates the density of growth observed. It will be noted that (as was also the case on solid medium) growth of all strains of *H. influenzae suis* was consistently less dense than that of human *H. para-influenzae* or of the Haemophilus-like organism. No strain examined appeared to require factor X. It was noted that although the Haemophilus-like organism grew well in the presence of factor V alone, growth was less good when both X and V were present; this apparent slight inhibitory effect of factor X was consistently observed with the Haemophilus-like organism.

Fermentation reactions. All strains tested failed to ferment the following 'sugars':—arabinose, rhamnose, galactose, lactose, trehalose, raffinose, inulin, glycerol, dulcitol, sorbitol, salicin, and inosite. The Haemophilus-like organism and human *H. para-influenzae* gave quite strong fermentation in two to three days with glucose, fructose, mannose, sucrose and mannitol; the Haemophilus-like organism also fermented xylose. By contrast, the Shope and Bakos strains of *H. influenzae suis* gave only very weak reactions at seven days, with glucose, sucrose, and maltose.

Serological Characteristics of the Haemophilus-like organism compared with those of Human Haemophilus para-influenzae *and of* Haemophilus-influenzae suis

The precipitation test was employed in these studies, using formamide extracts layered on sera prepared in rabbits.

Reaction of the Haemophilus-like organism strain 5408 *with other isolations from pigs of the same organism.* The serological relationship of the Haemophilus-like organism strain 5408 to seven other isolations of what was apparently the same organism from the lungs of pigs in the same herd was examined. Sera were prepared from all strains and each was cross-absorbed with strain 5408. It was found that an extract of strain 5408 precipitated strongly with the other sera, that extracts of the other strains reacted strongly with 5408 serum and that strain 5408 and all the other strains cross-absorbed each other completely. It was concluded, therefore, that, as a group, these strains were serologically identical.

Reaction of the Haemophilus-like organism strain 5408 *with four human strains of* Haemophilus para-influenzae. Two apparently different precipitating antigens were found in the four human strains of *H. para-influenzae* examined; three of the strains reacted with, and cross-absorbed, each other (429, 678 and 759), but the remaining strain (655) appeared to have an antigen specific to itself. When compared with the Haemophilus-like organism strain 5408, no reaction occurred between an extract of 5408 and the human strain sera, nor between human strain extracts and 5408 serum. A serological difference between the Haemophilus-like organism and the other strains appeared, therefore, to exist.

Reaction of the Haemophilus-like organism strain 5408 *with one Shope strain and four Bakos strains of* Haemophilus influenzae suis. An extract of strain 5408 failed to precipitate with sera of the Shope strain 3510/14 and of the Bakos type strains A, B, C and D. Likewise, extracts of the *H. influenzae suis* strains gave no reaction with 5408 serum. Therefore, there appeared to be a serological difference between the Haemophilus-like organism and the five *H. influenzae suis* strains. Further, the four Bakos type strains appeared on cross-absorption tests to be distinct from each other thus confirming Bakos's report on these strains.

Throughout this work there was no indication of the existence of a precipitating antigen common to all—or a majority—of the Haemophilus strains examined. It was noted that satisfactory antigens for the precipitation test could be prepared from the Haemophilus-like organism in either its rough or smooth phase of growth.

The final conclusion was reached that, on the basis of a precipitation test, the Haemophilus-like organism was serologically distinct from the other Haemophilus strains examined.

Pathogenicity for Pigs of the Haemophilus-like Organism and of Haemophilus influenzae suis.

It was suggested in the Introduction that part of the explanation

for the apparent low-grade pathogenicity of the Haemophilus-like organism originally isolated might be that its poor growth on artificial media made it difficult to obtain a reasonably large number of viable organisms for pig inoculation. The organism produced pneumonia and pleurisy, however, when inoculated into pigs along with swine fever virus. The new strain of Haemophilus-like organism produced pneumonia and pleurisy in pigs when a large number of viable organisms, without the addition of swine fever virus, was inoculated. If it could be shown that a non-pathogenic dose of the new strain could he made pathogenic by the addition of swine fever virus to the inoculum, the indications would be that these two organisms were pathogenically similar.

TABLE 2

INTRA-TRACHEAL PATHOGENICITY FOR PIGS

Various dilutions of the Haemophilus-like organism strain 5408 and of undiluted culture of *Haemophilus influenzae suis* strain 3510/14.

Inoculum	*Dilution of culture*	*Pig No.*	*Killed: days after inoc.*	*Histological abnormality*	*Organism isolated*
Haemophilus-like organism	Undiluted	1	7	Yes*	Haemophilus-like organism
,,	10^{-3}	2	14	,,	,,
,,	10^{-5}	3	7	,,	,,
,,	10^{-5}	4	14	,,	,,
,,	10^{-7}	5	6	No	None
,,	10^{-7}	6	7	,,	,,
,,	10^{-7}	7	8	,,	,,
,,	10^{-7}	8	8	,,	,,
,,	10^{-7}	9	14	,,	,,
,,	10^{-7}	10	14	,,	,,
Haemophilus influenzae suis	Undiluted	11	7	No	None
,,	,,	12	7	,,	,,
,,	,,	13	7	,,	,,
,,	,,	14	7	,,	,,
,,	,,	15	7	,,	,,
,,	,,	16	7	,,	,,
,,	,,	17	7	,,	,,
,,	,,	18	14	,,	*H. suis*
,,	,,	19	14	,,	None

*The changes described by Pattison *et al.* (1957) for their Haemophilus-like organism, including pleurisy.

Two series of pig inoculations were carried out. In the first a non-pathogenic dose of the new strain of the Haemophilus-like organism was determined, and the histological lesions produced by pathogenic doses were compared with those previously observed with the original strain of the Haemophilus-like organism. At the same time large intratracheal inocula of *H. influenzae suis* (Shope strain, 3510/14) were made into other pigs in order to compare the reactions to this organism and to the Haemophilus-like organism. The results

are given in Table 2, from which it will be seen that a dilution of 10^{-7} viable organisms (approximately 1,000) of the new Haemophilus-like organism consistently failed to produce a reaction, and that the largest number of viable *H. influenzae suis* inoculated (approximately 3,000 million organisms) failed to produce disease. *H. para-influenzae,* strain 5408, has maintained its virulence for pigs through many laboratory subcultures over a period of two years. This negative result with *H. influenzae suis* strain 3510/14 might not apply to all strains of this organism, especially if they were freshly isolated. Such strains were not available, however, to test this possibility.

In the second series of inoculations, swine fever virus was added to the previously determined non-pathogenic dose of the new Haemophilus-like organism, and also to the same inoculum of *H. influenzae suis* as used in the first series of inoculations. All animals inoculated with virus developed classical swine fever. The results are given in Table 3.

TABLE 3

INTRA-TRACHEAL PATHOGENICITY FOR PIGS

A non-pathogenic dose of the Haemophilus-like organism strain 5408, or of *Haemophilus influenzae suis,* when injected with swine fever virus.

Inoculum	*Dilution of culture*	*Pig No.*	*Killed–days after inoc.*	*Histological abnormality**	*Organism isolated*
Haemophilus-like organism	10^{-7}	20	6	H	Haemophilus-like organism
,,	10^{-7}	21	7	S	Coagulase-negative staphylococcus
,,	10^{-7}	22	8	SF	None
,,	10^{-7}	23	8	,,	,,
Haemophilus influenzae suis	Undiluted	24	7	SF	None
,,	,,	25	7	,,	,,
,,	,,	26	8	,,	,,
,,	,,	27	8	,,	,,

*H The changes described by Pattison *et al.* (1957) for their Haemophilus-like organism, including pleurisy.

S Multiple staphylococcal nodules.

SF Haemorrhages and collapse associated with swine fever virus; no pleurisy.

In one case out of four, the swine fever virus enhanced the pathogenicity of the small number of Haemophilus-like organisms inoculated, to the extent of causing extensive pneumonia and pleurisy that were indistinguishable from the lesions produced by Pattison *et al.* (1957) or by large inocula of the Haemophilus-like organism without swine fever virus. The inocula of *H. influenzae suis* remained non-pathogenic even when they contained swine fever virus.

In these experiments the histological lesions found in pigs inoculated with the new strain of the Haemophilus-like organism were in every way identical with those previously described by Pattison *et al.* They varied in extent from isolated nodules, usually situated in the dorsal region of the diaphragmatic lobes and with an overlying pleurisy in pigs inoculated with relatively small numbers of viable organisms (e.g. Table 2, pigs 3 and 4 that received 10^{-5} viable organisms), to extensive pneumonia with pleurisy in pigs given large numbers of viable organisms. The constant presence of pleurisy in association with the Haemophilus-like organism, as reported by Pattison *et al.*, was again noted. No similar histological lesion was found in any pig inoculated with *H. influenzae suis,* either alone or along with swine fever virus.

Identification of the Haemophilus-like Organism

On the basis of the cultural and biochemical characteristics given above, the conclusion was reached that the Haemophilus-like organism differed from *H. influenzae suis* and that it could be included among those members of the Haemophilus genus that grow in the absence of factor X. The strain was sent to Dr. Margaret Pittman who kindly identified it as belonging to the *H. para-influenzae* group. Serologically the organism differed from all the strains of *H. influenzae suis* examined. No cross precipitation was obtained with three strains of *H. para-influenzae* of human origin, and the possibility exists that this pig strain of *H. para-influenzae* is serologically distinct. Strong serological reactions of identity were obtained between the strain 5408 used in this work and seven other isolations of what was apparently the same organism from pigs in the same herd as that from which strain 5408 was isolated. The experimental pathogenicity of *H. para-influenzae* strain 5408 was different from that of *H. influenzae suis* strain 3510/14, but the histological lesions produced by the original Haemophilus-like organism and by strain 5408 were identical.

It was finally concluded, therefore, that the original Haemophilus-like organism and the new strain were the same, that they differed from *H. influenzae suis*, and that they could properly be named *H. para-influenzae*.

DISCUSSION

On the basis of the observations recorded by Pattison *et al.* (1957) and those reported here, it would seem that two members of the genus Haemophilus occur in the lungs of pigs in Britain. *H. influenzae suis* has been identified quite frequently, but attention does not appear to have been drawn to *H. para-influenzae*, as defined above.

The occurrence of a characteristic histological picture and the constant presence of pleurisy should be of considerable assistance in recognising disease associated with *H. para-influenzae.* These indications of the presence of this organism may be particularly useful if further strains similar to that originally examined are encountered,

because in these cases cultural identification may be difficult.

It will be noted from Table 1 that the seven strains of *H. influenzae suis* tested did not apparently require X factor for growth. This is contrary to the original description of the organism given by Lewis and Shope (1931) and Shope (1931). However, Alexander (1958) has reported that some strains do not require factor X and that the essential growth needs of *H. influenzae suis* have not yet been defined. This being so, the differentiation of *H. influenzae suis* from *H. para-influenzae*, the Haemophilus-like organism described in this paper, becomes increasingly difficult. Indeed, in the present state of our knowledge, it would be impossible with certain strains to differentiate between these two organisms on the basis of their growth factor requirements alone. The indications are that a serological method of identification using the precipitation test as described here might be of considerable assistance.

Thus, the factors to be considered in the final differentiation between *H. influenzae suis* and *H. para-influenzae* isolated from pigs would be (a) growth factor requirements (b) fermentation reactions with certain 'sugars' (c) serological reactions with specific sera (d) density of growth in suitable media and (e) histopathological lesions in the pigs from which the organisms were isolated.

CONCLUSIONS

A description is given of the cultural characteristics, serological reactions and pathogenicity for pigs of an organism of the Haemophilus genus isolated from pigs showing lesions of pneumonia and pleurisy. The conclusion is reached that the organism differs from *Haemophilus influenzae suis* and is a member of the *Haemophilus para-influenzae* group, and that it is probably the same organism as that originally described by Pattison, Howell and Elliot (1957).

ACKNOWLEDGMENTS

We are most grateful to Mr. J. Elliot, Manager of the Isolation Compound, for arranging the husbandry of the experimental pigs, and to Miss A. Fisher and Mr. G. C. Millson for technical assistance.

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THE HISTOLOGY OF THE SPINAL AND SYMPATHETIC GANGLIA AND THE ADRENAL GLANDS IN SCRAPIE DISEASE OF SHEEP

By

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INTRODUCTION

There are few reports on the histology of the spinal sensory or sympathetic ganglia in scrapie.

Besnoit and Morel (1898) claimed that the spinal ganglia were congested and infiltrated secondary to peripheral neuritis. They were examined by Stockman (1926) who observed intracytoplasmic eosinophilic inclusions, and by Brownlee (1940), who saw vacuoles in the neurones of both scrapie and control sheep. A few sympathetic ganglia have been examined by Brownlee (1940), who observed no abnormalities apart from one vacuole in a scrapie sheep, but Godina (1950) has recorded vacuolation in healthy sheep.

Adrenal glands have been examined by Weihermüller (1939) who observed an inconstant variation in the relative sizes of the zonae reticularis and fasciculata, and by Brownlee (1940) who found that in seven out of ten scrapie sheep the adrenals were normal, while the remining three showed non-specific changes. Bosanquet, Daniel and Parry (1956) stated that the adrenals were enlarged in the terminal stages of the disease.

The occurrence of vacuoles in the central nervous system is considered of histopathological significance in scrapie. This communication reports a study of the comparative histology of the ganglia and adrenal glands of healthy and scrapie sheep, with particular reference to the occurrence of vacuoles.

MATERIALS AND METHODS

Ganglia were obtained from thirty sheep affected with natural scrapie, ten in which scrapie had been experimentally induced with an inoculum originating from the transmission experiments of Wilson, Anderson and Smith (1950) and ten healthy control sheep. Spinal ganglia were from the cervical region and cervical intumescentia (C_4, C_7 and C_8), the mid-thoracic and upper lumbar region (T_7, T_8, L_2 and L_3), and the lumbo-sacral intumescentia (L_6 and S_1). The sympathetic ganglia were the stellates, the ganglia of the thoracic trunk and the coeliaco-mesenteric ganglia. The numbers of vacuoles present were counted in 48 sections of these spinal and 48 sections of sympathetic ganglia from each animal. The Gasserian, anterior cervical sympathetic and posterior mesenteric ganglia were also examined but vacuoles were not counted. The histological techniques used were similar to those previously reported (Wight, 1960).

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Both adrenal glands were obtained from fifty-seven sheep with natural scrapie, thirteen with experimental scrapie and one hundred and twelve apparently healthy sheep. The majority of the sheep with the natural disease were between two and three years of age, and those with experimental scrapie were six months to twenty-one months of age. The control sheep were obtained from the Edinburgh abattoir and this institute: twenty-two were between six months and two years of age while the remainder were more than two years, many being cast ewes up to seven years old.

The adrenal glands were fixed in 10 per cent. formal saline prior to processing and paraffin embedding. Sections were stained with haematoxylin and eosin, Van Gieson, and Carbol chromatrope (Lendrum, 1944), and in selected cases with Gordon-Sweet's reticulin stain, Ziehl-Neelsen's carbol fuchsin, von Kossa's stain for calcium, Masson's trichrome, Sudan IV, Gomori's aldehyde fuchsin, the periodic acid-Schiff method, and the ferric ferri-cyanide method of Schmorl.

RESULTS

Spinal and Sympathetic Ganglia

No characteristic macroscopic changes were present in the spinal or sympathetic ganglia of the scrapie sheep.

The commonest microscopic change was vacuolation of the cytoplasm of neurones, but these vacuolated neurones were not significantly more numerous in either spinal or sympathetic ganglia of the scrapie groups than in the healthy control sheep. In spinal ganglia they were observed in 90 per cent. of controls, 70 per cent. of experimentals, and 83·3 per cent. of natural cases of scrapie; the average number of vacuoles in 48 sections being 6·2 (range 0 to 15) in the controls, 5·6 (range 0 to 19) in the experimentals, and 6 (range 0 to 18) in the natural cases. In the sympathetic ganglia, vacuoles were seen in 66 per cent. of control sheep, 50 per cent. of experimental cases, and 70 per cent. of natural cases, and the average number of vacuoles per case was 1·8 (range 0 to 7) in the controls, 0.6 (range 0 to 2) in the experimentals, and 2.1 (range 0 to 10) in the natural cases of the disease. Vacuoles were also present in the Gasserian ganglia, the anterior cervical sympathetic and posterior mesenteric sympathetic ganglia, and were apparently not more numerous in those from scrapie sheep than from the controls.

In both types of ganglia the majority of the vacuoles were unilocular. They were of varying sizes; one very large vacuole in a spinal ganglion of a control animal measured 138 x 99 microns. In the spinal ganglia, vacuoles often contained eosinophilic material (Fig. 1) although this was usually in the form of an eosinophilic network or a diffuse mass of fine granules, rather than as distinct spheroids such as are seen in scrapie vacuoles in the central nervous system. Apart from this material, no specific substance, including fat, lipoid, carbohydrate and nucleic acid could be demonstrated (Fig. 2). The affected neurones were otherwise quite normal in appearance.

Apart from vacuolation, no significant abnormality was observed in the spinal or sympathetic ganglia. Rare chromatolytic and necrotic neurones and occasional neuronophagia and proliferation of amphicytes were seen in all groups. Neurones with pale cytoplasm and peripherally prominent Nissl substance were often seen in the sympathetic ganglia of all groups of sheep. Spheroidal hyaline masses sometimes lay close alongside the neurones of both scrapie and control sheep. Some seemed to be formed by the detachment of a necrotic piece of the cytoplasm of the neurone, while a study of silver preparations suggested that they may sometimes have been large end bulbs.

In nearly all animals an occasional nucleus in the sympathetic ganglia contained two nucleoli, but in two natural cases of scrapie intranuclear refractile bodies, faintly visible without staining, were present in a small number of cells in some ganglia of the sympathetic thoracic trunk. These stained strongly with haematoxylin (Fig. 3) but not with Einarson's gallocyanin at pH 1·64. Intracytoplasmic lipochrome granules and phloxinophilic bodies similar in histochemical reactions to those described in the central nervous system of sheep (Zlotnik, 1957; Wight, 1960), were present in both spinal and sympathetic ganglia and there was no difference in their occurrence in any group of sheep. Extracellular lipochrome was quite often seen in sympathetic ganglia.

Adrenal Glands

There was no constant difference in macroscopic appearance between the adrenals of the scrapie and control sheep when age, sex, and size of the breed and individual were taken into account.

The most striking histological appearance was the presence of aggregations of eosinophils in the adrenal medulla. These cells were morphologically similar to those in peripheral blood, and were present as small or large foci, frequently lying close to the central vein, and nearly always confined to the medulla. Sometimes the aggregations were so large that they almost completely replaced the chromaffin cells and in such cases the mass of eosinophils was easily visible macroscopically as a greenish-yellow, caseous core (Fig. 4). In such cases the reticulin network was richer but more delicate than normally. Usually both glands were affected.

Foci of eosinophils were seen in 52·6 per cent. of all the control sheep. However, 22 of the latter were less than two years of age and only 4·5 per cent. of these contained foci, whereas foci were seen in 64·4 per cent. of the remaining controls which were more than two years old. A similar eosinophilia was present in the adrenals from 10·5 per cent. of the natural cases of scrapie, but in none from the experimental cases.

Collections of cells resembling lymphocytes were also seen in the medulla of adrenals from 26·7 per cent. of the control sheep and 20 per cent. of those with natural scrapie. As many as three focal or

diffuse aggregations were seen in a single section and they were of small or medium size, but never as large as the masses of eosinophils.

As well as islets of misplaced cortical cells which were often found near the central vein, macrophages were also present in the medulla. In haematoxylin and eosin stained preparations, the cytoplasm of these macrophages was sometimes very enlarged, contained orange-yellow coloured material and often a few small basiphilic granules. This orange-coloured material was visible as golden yellow pigment in unstained sections. It stained only faintly orange-yellow with Sudan IV in paraffin sections, was acid fast, strongly P.A.S. positive, and stained rather irregularly with Gomori's aldehyde fuchsin as if only a part of the material was taking the stain. The material gave a positive Schmorl's reaction.

Sympathetic ganglion cells were seen in the adrenal medulla from five cases of natural scrapie, and in one case a small number of neurones were vacuolated. Neurones were not observed in adrenals from the experimental cases, but they were seen in 19 of the control sheep and in 10 of these vacuolated neurones were present. In one control animal 3 of the 9 neurones seen contained vacuoles, and in another control 11 out of 46 neurones visible were vacuolated. The vacuoles were usually monolocular and occasionally contained a little eosinophilic material. Apart from the vacuole, the neurones were morphologically unaltered in both scrapie and control sheep.

Small areas of fibrosis were not infrequent in the older sheep and in one control animal there was extensive replacement of the cortical parenchyma by fibrous tissue. Small necrotic foci, around which were masses of pigment material similar to that described above, were seen in adrenals from two control sheep. No other abnormalities were observed, and by the methods of examination used no significant difference was found between the adrenals of the scrapie sheep and the controls.

DISCUSSION

In the present study vacuoles were found in ganglia from a large proportion of both the scrapie and the control groups of sheep. The similar incidence in both groups indicates that vacuolation in the spinal and sympathetic ganglia is not significant in scrapie. These findings are of interest because of the importance attached to vacuolation of neurones in the histopathology of the brain stem and spinal cord (Bertrand, Carré and Lucam, 1937; Holman and Pattison, 1943; Palmer, 1957a; Zlotnik, 1957; 1958 a & b; Wight, 1960).

In the spinal ganglionic neurones of man, cat and rat, vacuoles containing lipoids have been associated with senescence (Truex and Zwemmer, 1942), but the vacuoles observed by Field (1952) in the spinal ganglia of 70 per cent. of normal rabbits contained neither lipoids nor glycogen. The latter did not find vacuoles in the spinal ganglia of normal sheep. The vacuoles now found in the spinal and

sympathetic ganglia of sheep resembled morphologically those seen by Field in the rabbit, and, as in the C.N.S. in scrapie, no specific contents could be demonstrated. However, in both the rabbit and in the scrapie and control groups of sheep the vacuolated neurones of the ganglia appeared otherwise unaltered, whereas in the C.N.S. in scrapie many vacuolated neurones were degenerated (Zlotnik, 1958a & b; Wight, 1960). This distinction from the degenerated vacuolated neurones in the C.N.S. in scrapie is as important as the increased numbers of vacuoles which occur in the disease, because it suggests that vacuolated neurones in ganglia may be physiological, while the degenerating vacuoles in the C.N.S. in scrapie are pathological. A similar interpretation must be placed on the vacuoles in the otherwise unaltered neurones of the adrenals of both scrapie and healthy sheep. Certain parts of the nervous system seem to show this physiological vacuolation more than others. Thus apart from spinal and sympathetic ganglia and adrenal glands, vacuoles may be found in moderate numbers in the medulla of clinically healthy sheep (Zlotnik and Rennie, 1957), whereas they are very rare in the healthy mesencephalon, pons, and medulla cord junction (Zlotnik, 1958a) and spinal cord (Wight, 1960).

According to Köhler (1952), the number of vacuoles in sympathetic ganglia is significantly increased in various conditions such as foreign body peritonitis in the bovine or colic in the horse, which suggests that an increased incidence in only an occasional case of scrapie might be non-specific.

Palmer (1957b) found eosinophilic spheroids in vacuolated medulla neurones of scrapie sheep, and similar structures were seen in vacuoles in the medullas of healthy sheep (Zlotnik, 1957). The eosinophilic material now found in the vacuolated neurones of sympathetic and spinal ganglia, and adrenal glands from both scrapie and control sheep is perhaps of a similar nature to that seen in healthy medullas. The rather different morphology may be due to factors other than disease.

Infiltration and congestion of the spinal ganglia as reported by Besnoit and Morel (1898) were not observed in the present study. Although there were occasional degenerating neurones in both control and scrapie sheep, significant morphological differences between the three groups were completely absent. This strongly indicates that lesions of the spinal ganglia were not a direct cause of the pruritus in scrapie.

Similar minor changes were found in sympathetic ganglia. Hyaline eosinophilic masses lying alongside the sympathetic ganglionic neurones of the horse have been thought to have pathological significance by Köhler (1952), but they were infrequent in the present study. Multiple intranuclear bodies occurred in such a small proportion of cases that they cannot be related to scrapie with certainty. In man, many changes in sympathetic ganglia are non-specific variations, which, according to Kuntz (1938), can only be

considered patholgical when marked or present in considerable numbers.

Stokoe (1959) found eosinophils in approximately 6 per cent. of 250 adrenals which he examined, whereas in the present survey they were found in 52.6 per cent. of healthy sheep. This considerable difference may be associated with age, for the majority of sheep used by Stokoe (1958) were between 7 and 11 months old, whereas in the present study the majority of controls were over 2 years old, many being cast ewes of 5 to 7 years. Eosinophils were found in 4·5 per cent. of the 22 sheep under 2 years, which is not greatly different from the proportion observed by Stokoe. The majority of the natural scrapie sheep were between 2 and 3 years old, thus lying somewhere between the younger group and the older group of control sheep in respect of the ages of the groups.

There was no evidence that the foci of eosinophils or lymphocyte-like cells were associated with scrapie disease. The golden-yellow pigment and the basiphilic granules in the cytoplasm of macrophages in the medulla were probably derived from the lipids and nuclei of degenerated cortical cells. Cohrs (1952), refers to increased lipofuscin deposition in the Zona Reticularis of older animals of several species.

CONCLUSIONS

Vacuolated neurones, which were otherwise of normal morphology, were observed in the spinal and sympathetic ganglia of a large proportion of both healthy and scrapie sheep. The vacuolated neurones were neither dissimilar in appearance nor more numerous in the ganglia of scrapie sheep. No specific contents were demonstrable in the vacuoles.

There was no significant morphological change in the spinal or sympathetic ganglia in scrapie disease, which suggests that the symptoms are not caused by lesions in these ganglia. The available evidence indicates that they are due to lesions in the central nervous system.

No difference was found between the adrenal glands of healthy sheep and those with scrapie. Vacuolation of otherwise unaltered neurones occurred in both groups. An eosinophilia of the medulla was also found in both groups and was particularly common in older sheep.

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I wish to thank Dr. J. T. Stamp, Director of the Moredun Institute, for the interest he has taken in this study, Dr. I. Zlotnik for his advice and guidance during the course of the work and Mr. J. C. Rennie for his technical assistance.

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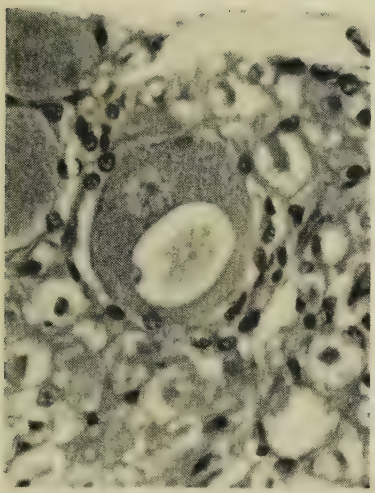

FIG.I

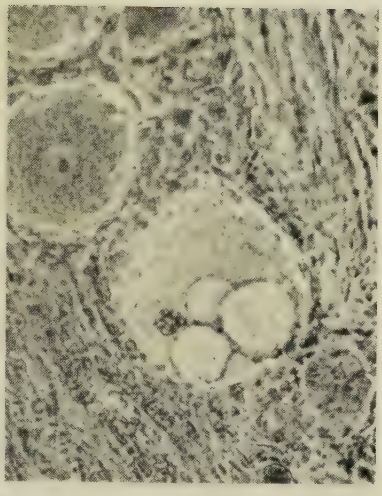

FIG.II

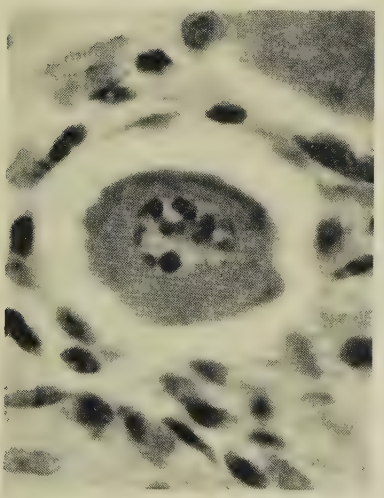

FIG.III

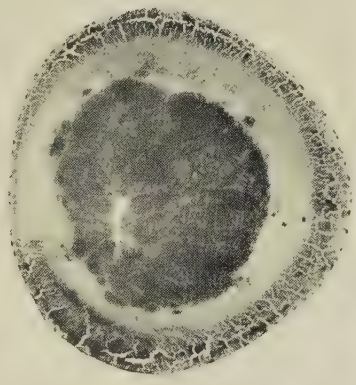

FIG.IV

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Vacuole containing eosinophilic material in an otherwise normal neurone from a spinal ganglion of a healthy control sheep. H. & E. x 500.

Fig. 2. Absence of fat in a multilocular vacuole in a neurone from a spinal ganglion of a healthy control sheep. Sudan IV/haematoxylin. Frozen section. x 330.

Fig. 3. Multiple intranuclear inclusions in a sympathetic ganglion from a case of natural scrapie. Haematoxylin/Phloxine/Tartrazine. x 870.

Fig. 4. Large mass of eosinophils almost completely replacing the medulla of the adrenal gland of a healthy control sheep. Carbol/chromatrope. x 8.

METABOLIC CHANGES IN CATTLE NEAR THE TIME OF PARTURITION

II. Hepatic Fat and Glycogen Content, together with Glucose-6-phosphatase, Phosphorylase and β-glucuronidase Activity of Liver

By

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INTRODUCTION

The appearance of stainable fat and an increase in alkaline phosphatase activity of bovine liver near parturition were described in the first communication of this series (Ford, 1959). It was thought desirable to see whether the increase in fat content could be confirmed on a quantitative basis and also to see whether information on the significance of the raised alkaline phosphatase activity could be obtained by the study of a substrate—specific phosphatase which was known to have a definite function in tissue metabolism. For this purpose glucose-6-phosphatase was chosen. Liver glycogen content was studied because of the low levels found in cattle with acetonaemia and phosphorylase activity because of its importance in glycogen breakdown. β-glucuronidase levels were studied following the finding of Bernard and Odell (1950) that an increase in liver activity occurs in the parturient rat.

MATERIALS AND METHODS

Animals and sampling. The management of the cattle used was described in the previous paper. Liver biopsy was carried out at intervals of seven to twenty days commencing two to three months before the expected date of calving and continuing up to about three months after parturition. The sample of liver was placed on filter paper to allow drainage of blood and divided into three portions for weighing. A portion of up to 200 mg. was homogenised in 3 ml. of distilled water for enzyme assay. About 50 mg. was homogenised in 2 ml. of cold 5 per cent. trichloracetic acid (T.C.A.) and placed in the refrigerator, whilst the remainder of the liver was dried overnight in an oven at 100°C.

Analytical methods. The dried liver sample was extracted in a Soxhlet apparatus with diethyl ether for 16 hours and the extracted lipid weighed. The cold 5 per cent. T.C.A. homogenate was centrifuged at 3000 R.P.M. for 10 minutes, the supernatant was removed and the deposit re-extracted twice with cold 5 per cent. T.C.A. The combined extracts were made up to 10 ml. and the glycogen content of an aliquot was determined with anthrone according to the method of Carroll, Longley and Roe (1956).

Glucose-6-phosphatase activity of the water homogenate was assayed by a modification of the method of Duve, Berthet, Hers and Dupret (1949). The substrate was 0·025 M glucose-6-phosphate and the buffer maleic acid—sodium maleate, pH 6·5. Test mixtures, substrate and

homogenate blanks were incubated at 37°C for 30 min. and the reaction stopped by the addition of 10 per cent. T.C.A. Liberated inorganic phosphate was estimated by the method of Fiske and Subba Row (1925) and enzyme activity was expressed as mg. P liberated in 30 min. per gramme of wet liver. Phosphorylase activity in the water homogenate after mixing with an equal quantity of 0·1 M sodium fluoride was assayed according to the method of Sutherland (1955) using 0·05 M glucose-1-phosphate as substrate in the presence of glycogen and adenosine-5′-monophosphate. Inorganic phosphate liberated after 10 min. incubation at 37°C. was estimated, and enzyme activity expressed as mg P liberated in 10 min. per gramme wet liver.

0·01 M phenolphthalein β-glucuronide was used as substrate for β-glucuronidase assay in the presence of 0·1 M acetate buffer at pH 5·2. After incubation at 37°C. for 1 hour, alkaline glycine of pH 10·7 was added and the free phenolphthalein determined colorimetrically. Walker and Levvy (1951) showed that binding of β-glucuronidase to the particulate fraction of homogenates may give rise to error in tissue assays. They described various measures for bringing the total activity into solution. Preliminary trials with bovine liver showed that there was no increase in activity following either pre-incubation with acetate buffer pH 5·2, long treatment in a Waring blendor or the addition of the surface-active agents, Teepol XL and Triton X100. Tenfold dilution of the original homogenate increased activity and therefore all assays of homogenised biopsy material were carried out in dilutions of 1:5, 1:10 and 1:25 and only results which were proportional to concentration in at least two of these dilutions were accepted. The unit of activity is the release of 1 μg. phenolphthalein in 1 hour at 37°C.

RESULTS

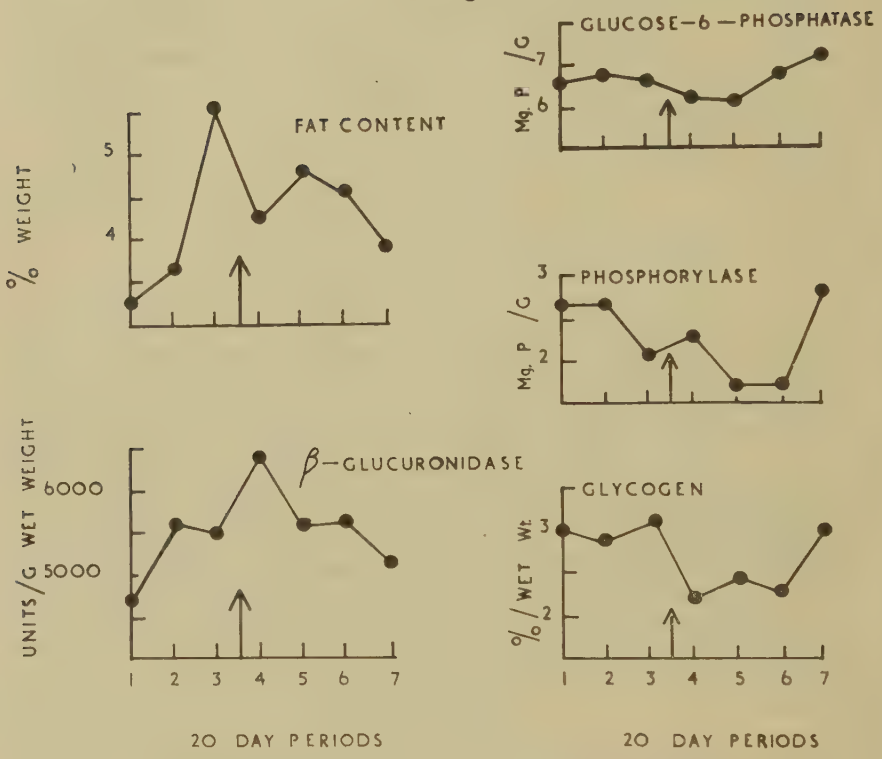
The results are detailed in Tables 1 to 6, and in Figures 1 to 3.

A total of 12 cows was used; fat and glucose-6-phosphatase levels were studied in 9 cows, phosphorylase and glycogen levels in 11 and β-glucuronidase levels in all 12 animals. In all cases the results from the individual biopsies were grouped in 20 day periods before and after parturition, and the mean values for these periods were compared. Fig. 1 shows the mean values for each 20 day period.

Fat Content

In the case of extractable fat (Table 1) the highest mean value occurred in the 20 day period immediately before parturition (Period 3), but statistical analysis showed that this mean was not significantly higher than the low levels in the 60 to 41 day period before parturition (Period 1) or in the 61 to 80 day period after parturition (Period 7). Examination of the histological assessment of hepatic fat in Table 3 of the previous paper (Ford, 1959) showed that the time of occurrence of maximum stainable fat varied from one week before to several weeks after parturition, and if the figures for extractable liver fat obtained in the present investigation are plotted against time (Fig. 2) the peak values of individual animals are seen to occur before, after or over a period including parturition.

Fig. 1



Mean values for bovine liver constituents during 20 day periods.

It was therefore necessary to see whether liver fat content during the whole of the period in which peaks occurred was significantly higher than during the periods before and after that in which peaks occurred. In each individual cow a mean was obtained for the whole period 40 days before to 60 days after parturition and from this was subtracted the mean value for the periods 60 to 41 days before and 61 to 80 days after parturition.

In seven out of the eight cows for which complete figures were available this difference was positive and the mean increase for 8 cows was 1·55 ± 0·507 per cent. being significant at the 2 per cent. level.

Glucose-6-phosphatase

The activity of this enzyme showed no change associated with parturition, activity being fairly constant in the region of 6·1 to 7·3 units (Table 2). Values for individual animals showed no peaks and the differences between the means of periods 2 and 5 and of periods 5 and 7 are not significant.

TABLE 1

FAT CONTENT OF BOVINE LIVER IN RELATION TO PARTURITION

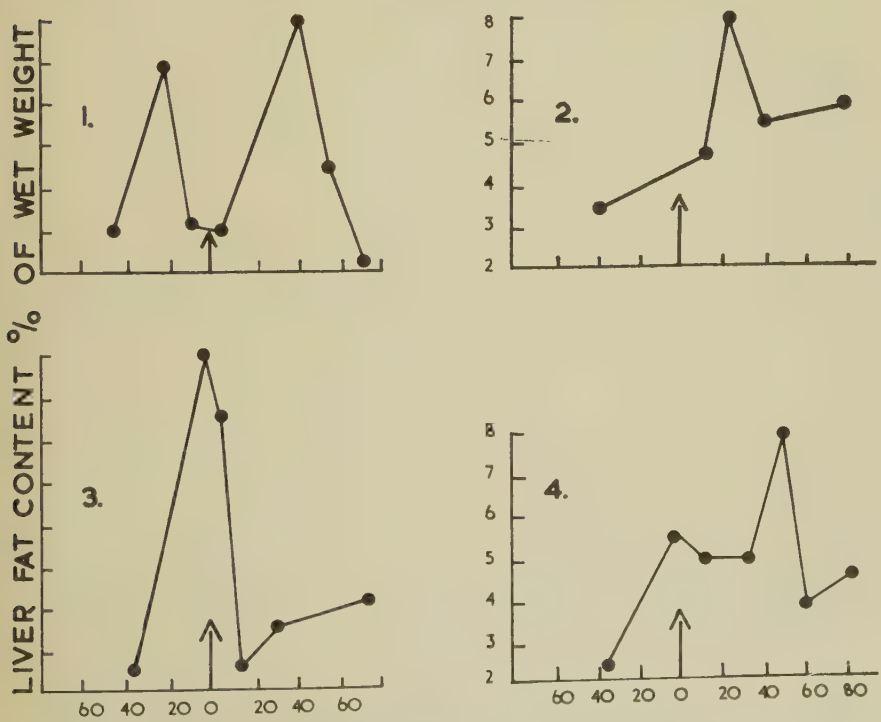
Percentage of wet weight

Period	1	2	3	4	5	6	7
Days from parturition	60–41	40–21	20–1	1–20	21–40	41–60	61–80
Number of observations	6	6	9	10	8	7	11
Mean	3·26	3·65	5·54	4·23	4·80	4·54	3·84
S.E.M.	± 0·61	± 0·84	± 0·78	± 0·65	± 0·95	± 0·70	± 0·71

Comparison of periods

Periods compared	D.F.	t	P
1 and 3	13	1·94	< 0·1
3 and 7	17	1·7	< 0·2

Fig. 2



Examples of liver fat changes in individual animals.

TABLE 2

GLUCOSE-6-PHOSPHATASE ACTIVITY OF LIVER

mg.P liberated/g. wet weight/30 min.

Period	1	2	3	4	5	6	7
Days from parturition	60–41	40–21	20–1	1–20	21–40	41–60	61–80
Number of observations	6	15	13	14	11	8	11
Mean	6·6	6·8	6·6	6·3	6·1	6·8	7·3
S.E.M.	± ·61	± ·70	± ·53	± ·64	± ·66	± ·46	± ·46

TABLE 3

PHOSPHORYLASE ACTIVITY OF LIVER

mg.P liberated/g. wet weight/10 min.

Period	1	2	3	4	5	6	7
Days from parturition	60–41	40–21	20–1	1–20	21–40	41–60	61–80
Number of observations	9	16	18	16	15	13	16
Mean	2·68	2·70	2·14	2·32	1·76	1·71	2·86
S.E.M.	± 0·39	± 0·47	± 0·24	± 0·41	± 0·32	± 0·21	± 0·45

Comparison of periods

Periods compared	D.F.	t	P
2 and 3	32	1·11	< 0·3
4 and 5	29	1·09	< 0·3
6 and 7	27	2·07	< 0·05
2 and 5	29	1·69	< 0·1

Phosphorylase

Table 3 shows that phosphorylase activity of approximately 2·7 units in the 60 to 21 day period before parturition fell in two stages to levels of 1·7 units in the 20 to 60 day period after parturition and then rose in the subsequent 20 days to slightly more than the pre-calving level. This latter rise was significant at the 5 per cent. level,

but the two stages in the fall in activity were not statistically significant when taken separately. If however the results are considered in 40 day periods (Table 4), the difference between the mean value of 2·69 ± 0·32 units for the 60 to 21 day period pre-partum and the mean value of 1·74 ± 0·09 units for the 21 to 60 day period post-partum is significant at the 0·1 per cent. level.

TABLE 4

PHOSPHORYLASE ACTIVITY OF LIVER

mg.P liberated/g. wet weight/10 min.
Results in 40 day periods

Period	1 & 2	3 & 4	5 & 6
Days from parturition	60–21	20 before to 20 after	21–60
Number of observations	25	34	28
Mean	2·69	2·22	1·74
S.E.M.	± 0·32	± 0·23	± 0·09

Comparison of periods

Periods compared	D.F.	t	P
1 & 2 with 3 & 4	57	1·23	< 0·2
3 & 4 with 5 & 6	60	1·6	< 0·1
1 & 2 with 5 & 6	51	7·1	< 0·001

Glycogen

Table 5 shows that glycogen content of about 3 per cent. before parturition falls to less than 2·3 per cent at parturition and rises to almost 3 per cent. in the 61 to 80 day period after parturition. This fall and rise are significant at the 1 per cent. and 2 per cent. levels respectively. If the mean value of 3·03 ± 0.13 per cent. for the 60 days before parturition is compared with the mean of 2·32 ± 0·13 per cent for 60 days after parturition the difference is significant at the 0·1 per cent. level.

β-glucuronidase

In Table 6 the mean β-glucuronidase level is seen to rise to a peak in the 1 to 20 days after calving compared with the levels in the 60 to 41 days before or after calving, yet these differences are

TABLE 5
GLYCOGEN CONTENT OF LIVER

Percentage of wet weight

Period	1	2	3	4	5	6	7
Days from parturition	60–41	40–21	20–1	1–20	21–40	41–60	61–80
Number of observations	13	16	19	18	14	11	17
Mean	3·03	2·95	3·08	2·23	2·38	2·36	2·94
S.E.M.	± ·19	± ·27	± ·24	± ·20	± ·23	± ·25	± ·22

Comparison of periods

Periods compared	D.F.	t	P
3 with 4	35	3·28	< ·01
3 with 6	33	2·48	< ·02

Comparison of levels before and after parturition

	60 days before	*60 days after*
Number of observations	48	43
Mean value	3·03	2·32
S.E.M.	± 0·13	± 0·13
D.F.	89	
t	4·8	
P	< ·001	

TABLE 6
β-GLUCURONIDASE ACTIVITY OF LIVER

units / g. wet weight

Period	1	2	3	4	5	6	7
Days from parturition	60–41	40–21	20–1	1–20	21–40	41–60	61–80
Number of observations	10	16	22	19	16	15	14
Mean	4750	5675	5545	6363	5634	5590	5157
S.E.M.	± 245	± 490	± 376	± 692	± 400	± 400	± 292

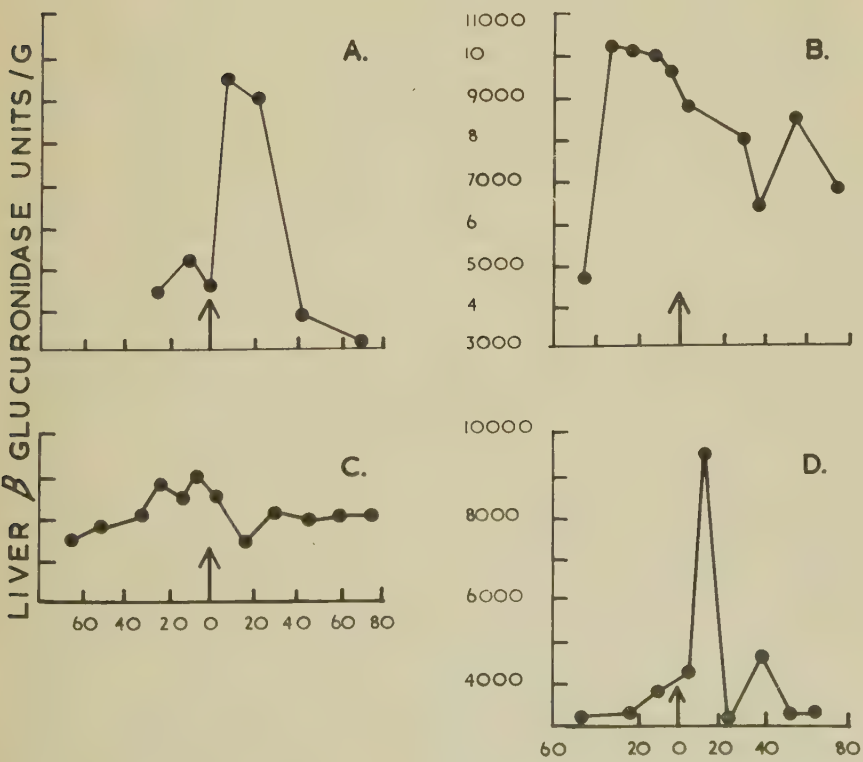
Comparison of periods

Periods compared	D.F.	t	P
1 with 4	27	1·63	< 0·2
4 with 7	31	0·47	< 0·7

not significant. As with the fat increases individual peaks may occur in a broad period including parturition (Fig. 3) and there are differences between control levels of individual cows.

The results were therefore examined in the same way as the fat results and it was found that in ten out of twelve cattle the mean activity for the period from 40 days before to 60 days after parturition was higher than the mean of levels during the 60 to 41 days before and the 61 to 80 days after parturition. For twelve cattle the mean increase in liver β-glucuronidase activity was 749 ± 331 units per gram wet weight being significant at the 5 per cent. level. There is, therefore, a significant peak in β-glucuronidase activity, but the peak varies in relation to parturition. This variation in the time of peak activity is illustrated by the curves for individual animals shown in Fig. 3.

Fig. 3



Examples of liver β-glucuronidase changes in individual animals.

DISCUSSION

Results obtained by extracting and weighing the ether soluble material from bovine liver before and after parturition have confirmed previous histological findings (Ford, 1959). Livers with no histologically detectable fat contain approximately 3 per cent. of ether soluble material. This figure is increased by varying amounts at the peak period, but the rise is small compared with the eightfold increase seen at the time of the onset of symptoms of acetonaemia (Ford and Boyd, 1960). Nevertheless, the findings of this investigation show that the same type of change, i.e. increase in fat content, occurring in acetonaemia also occurs to a lesser degree in the asymptomatic cow close to the time of parturition.

In the previous paper the difficulty of interpreting the significance of the rise of liver alkaline phosphatase activity in early lactation was emphasised. The assay is carried out at the relatively unphysiological pH of 9 and the substrate, phenyl phosphate, is unlikely to be available in appreciable quantities in the normal liver cell. For these reasons it was necessary to investigate the activity of a phosphatase which is active at a pH near neutrality and which also acts on a substrate known to be essential for cellular metabolism. The selected enzyme, glucose-6-phosphatase is responsible for the hydrolysis of glucose-6-phosphate to glucose and inorganic phosphate and thus controls the final stage in the production of free glucose whether as a result of glycogenolysis or by gluconeogenesis from propionate or protein. This investigation has shown a fairly constant level of hepatic glucose-6-phosphatase activity of about 6 mg. P liberated by 1 g. liver in 30 minutes, a level approximately six times greater than the activity of rat liver and twelve times higher than that of human liver (Weber and Cantero, 1957). This difference may be a reflection of the fact that the monogastric animals derive an appreciable part of their blood glucose by direct absorption from the intestine whereas the ruminant receives negligible amounts of hexose from this source and is therefore probably more dependent on glycogenolysis for the maintenance of blood sugar levels.

The ruminant may be comparable with the fasting non-ruminant in which Weber and Cantero (1957) have shown that liver glucose-6-phosphatase levels remain in the normal range during fasting for periods up to six days, although phosphohexose isomerase and phosphoglucomutase fall to one third of the prefasting levels. The absence of an increase in glucose-6-phosphatase activity compared with alkaline phosphatase activity is notable and it is clear that the factor or factors responsible for the increase in activity of the latter enzyme have no effect on the former.

The enzyme phosphorylase showed a significant fall in activity commencing before parturition and continuing into early lactation with a rise to precalving levels about 80 days after parturition. In the rat, Sutherland and Cori (1951) showed that a fall in activity follows adrenalectomy and is accentuated by fasting. The change in

phosphorylase activity is attributed by them to changes in the balance of enzymes which activate and inactivate phosphorylase and which are regulated by adrenalin and glucagon. The same workers have also shown that phosphorylase is the rate limiting enzyme in the breakdown of glycogen. Work by Mommaerts, Illingworth, Pearson, Guillory and Seraydarian (1959) has shown that in the absence of muscle phosphorylase glycogen breakdown does not occur, but synthesis is possible due to the activity of uridine diphosphate glucose-glycogen transferase. It seems likely, therefore, that the main physiological effect of a reduction in phosphorylase activity is reduced glycogenolysis which may be reflected in the low blood glucose levels which occur in early lactation.

Shaw and Leffel (1949) give bovine liver glycogen levels derived from the analysis of biopsy samples taken before and after parturition, but the significance of their figures is uncertain because of variations in the nutritional status of their animals. Leffel and Shaw (1954) examined animals described as liberally fed in the 10 day period post-partum and obtained liver glycogen content of 2·3 per cent. and fat content of 6·5 per cent.

The present study has shown that the level during the 60 days after parturition was significantly lower than during the 60 days before parturition, but the fall was slight compared with that which occurs in clinical cases of acetonaemia (Shaw and Leffel, 1949; Ford and Boyd, 1960). It is often postulated that the onset of acetonaemia at the time of increasing hexose requirements for milk synthesis is related to the low amounts of dietary hexose available to ruminants and the relatively small reserve available from tissue glycogen compared with the demands of mammary tissue. The animal must in some way compensate for the increased hexose requirement and presumably the first process is glycogenolysis followed by gluconeogenesis from protein. Lindsay (1959) has pointed out the smallness of the ruminant liver glycogen reserve in relation to mammary requirements, a view which is supported by this evidence of a fall in liver glycogen at the time of greatest demand. The factors which control the changes in the relative activity of the various metabolic pathways in the ruminant in response to changes in milk production are not clearly understood but it seems likely in view of the evidence of Sutherland and Cori (1951) that the change in phosphorylase activity may control the liver glycogen level and may itself be controlled by hormonal factors.

The increase in β-glucuronidase activity shown in Table 6 parallels that found in the rat by Bernard and Odell (1950). There is much evidence concerning the control of β-glucuronidase activity by hormonal factors (in particular by oestrogens) in the rat and other animals (Fishman, 1955), and it seems likely that the increase in oestrogen output believed to occur at parturition (Schofield, 1957) may be responsible for the increased enzyme activity. It is now thought that the synthesis of glucuronides is brought about by glucuronyl transferase

whilst β-glucuronidase is responsible for the hydrolysis of conjugated glucuronides (Levvy and Marsh, 1959). The purpose of this increased capacity of liver to hydrolyse glucuronides at this stage of the reproductive cycle is by no means obvious.

The previous evidence of changes in metabolism near parturition was reviewed by Ford (1958). The present work has confirmed by different methods the increase in fat and reduction in glycogen, and an increase in β-glucuronidase and reduction in phosphorylase activity have now been demonstrated whilst glucose-6-phosphatase activity has been shown not to change.

CONCLUSIONS

In the normal parturient bovine there is a significant increase in extractable liver fat and a significant fall in liver glycogen, and in phosphorylase activity. There is also a significant increase in hepatic β-glucuronidase activity whilst glucose-6-phosphatase activity does not change.

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FISSION PRODUCTS AND THE DAIRY COW

5. The Radiotoxicity of Iodine-131

By

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INTRODUCTION

The incident of October, 1957 involving contamination of agricultural land with iodine-131 released from the Windscale No. 1 pile drew attention to the lack of information on the radiotoxicity of this fission product to the cow. A considerable body of information was available from the U.S.A. on the radiotoxicity of the isotope to sheep but there was some doubt as to the validity of the direct extrapolation of results from the sheep to even such a closely related animal as the cow. This paper describes a small scale experiment designed to examine the effects of iodine-131 on dairy cows.

METHODS

Preliminary plan of the experiment. In a study of the acute toxicity of ^{131}I to sheep, Bustad, Morrow, Hackett, Marks and George (1955) found that a single dose of 5 mc., estimated to deliver a radiation dose of 14,000-36,000 rad‡ to the thyroid gland, produced mild to severe thyroid damage but no haematological changes, while a single dose of 15 mc., delivering a thyroid radiation dose of 33,000 to 75,000 rad, brought about extensive necrosis of the thyroid gland and a depression in the lymphocyte count. Russell, Martin and Wortley (personal communication) implied that a radiation dose of 1,000 rad to the cow's thyroid gland would be acceptable under certain conditions of emergency. It was therefore decided to administer orally sufficient ^{131}I to deliver radiation doses to the thyroid gland of 1,000, 10,000 and 100,000 rad, i.e. radiation doses which would be expected to produce no damage, minimal damage and severe damage, respectively, to the gland.

The radiation dose to the thyroid gland from ingested ^{131}I can be calculated as follows: Integrated dose (rad) =

$$\frac{Q \times f_w \times 3{\cdot}7 \times 10^7 \times 0{\cdot}19 \times 1{\cdot}6 \times 10^{-6} \times 8{\cdot}64 \times 10^4 \times t}{100 \times m}$$

where Q = quantity of ^{131}I ingested (in mc.),
f_w = fraction of Q going to the thyroid gland,
$3{\cdot}7 \times 10^7$ = disintegrations/sec./mc.,
$0{\cdot}19$ = mean energy of radiation of ^{131}I (in MeV.),
$1{\cdot}6 \times 10^{-6}$ = ergs/MeV.,
$8{\cdot}64 \times 10^4$ = secs./day,
t = average life of ^{131}I in thyroid gland (*i.e.* effective half-life/0·693) (in days),
100 = ergs/g./rad,
m = mass of thyroid gland (in g.)

* Seconded from M.R.C. Radiobiological Research Unit, Harwell, Berks.

‡ The rad is a unit of absorbed energy and is equivalent to the absorption of 100 ergs of energy per gram of tissue.

In making the initial calculations, values for f_w of 0·2 and for t of 8 days were taken on the basis of the results of Swanson, Lengemann and Monroe (1957); m was assumed to be 25 g. These calculations suggested that the amounts of ^{131}I needed to give the required radiation doses would be 1·4, 14 and 140 mc. In order to simulate the Windscale incident these amounts were given over a 14 day period as equal daily doses of 0·1, 1 and 10 mc. Later, dosage levels of 0·5, 2 and 5 mc./day were introduced.

Since f_w is not constant but exhibits a definite seasonal variation it was considered desirable to initiate dosing at different times of the year. January-February and May-June were chosen as times at which thyroid activity could be expected to be near maximal and minimal respectively. Four animals were dosed at each level, two in winter and two in summer.

Assessment of thyroid radiation doses. As more information became available during the course of the work it became apparent that the

TABLE 1

ESTIMATED RADIATION DOSES TO THE THYROID GLAND

Animal	*Date of initial dose*	*Total dose of ^{131}I (mc.)*	*Fraction of dose going to thyroid gland (f_w)*	*Estimated radiation dose to thyroid gland (rad)*
Mayflower Tinkerbell	8.1.59.	140	0·36	290,000
Nanette Ornament	18.6.58.	140	0·18	150,000
Goldfinch Rosemary III	28.5.59	70	0·18	70,000
Nora III Colleen III	4.2.60.	70	0·07	30,000
Jo Gwen	28.5.59.	28	0·18	30,000
Colleen II Butterfly	8.1.59.	14	0·36	30,000
Meta Wakeful	18.6.58.	14	0·18	15,000
Lupin Russet	7.1.60.	27	0·07	10,000
Lavinia Swanny	26.6.59.	7	0·18	7,000
Lovely V Bunty	4.2.60.	7	0.07	3,000
Sadie Verity Elspeth Madeline	11.9.58.	1·4	0.18	1,500

assumed values for f_w, t and m were incorrect. More accurate values for f_w and t were estimated from the results of thyroid monitoring of control animals after administration of a test dose of ^{131}I: t has been taken as 9 days, values for f_w are given in Table 1. The average weight of thyroid glands removed from control animals at slaughter was 15 g. Substituting the revised values in the equation used for the calculation of the thyroid radiation dose led to the estimated values given in Table 1.

Animals. Mature, non-pregnant Ayrshire-Red Poll cows in their first month of lactation were used. They were maintained on a diet consisting of approximately 30 lb. hay and commercial dairy nuts (2½ lb./gal. milk) divided into two equal feeds. The original diet was estimated to provide approximately 3,000 μg. iodine daily: the minimal requirement for a 1,000 lb. cow producing 40 lb. milk daily is given by Mitchell and McClure (1937) as 400 to 800 μg. daily. Unfortunately the composition of the dairy nuts was changed, without notification from the manufacturers, in October, 1959, potassium iodide then being added at the rate of 2g./ton. The effect was to increase the daily iodine intake to about 7,000 μg. and so to depress the uptake of ^{131}I by the thyroid gland (Table 1.)

The appropriate amount of carrier-free ^{131}I was given daily at 16.00 hr., immediately following the afternoon milking, in the form of sodium iodide absorbed on to anhydrous sodium phosphate in gelatin capsules. Blood samples were taken daily at 11.00 hr. from the mammary vein.

One cow from each group of four at the same oral dosage level was slaughtered approximately 2 months after dosing for post-mortem examination and the others were inseminated artificially on coming into oestrus and are to be held for as long as is economically possible. As calves are born the bull-calves are slaughtered for post-mortem examination and the heifer-calves are reared as normal animals.

Biochemical determinations. Determinations were made at gradually lengthening intervals of plasma protein-bound iodine (Grossman and Grossman, 1955)., cholesterol, calcium, inorganic phosphorus, urea, total serum protein, albumin and globulin.

Haematological determinations. Serial determinations were made of the red cell count, packed cell volume, haemoglobin, total and differential white cell counts, platelet count, corpuscular fragility, viscosity and specific gravity.

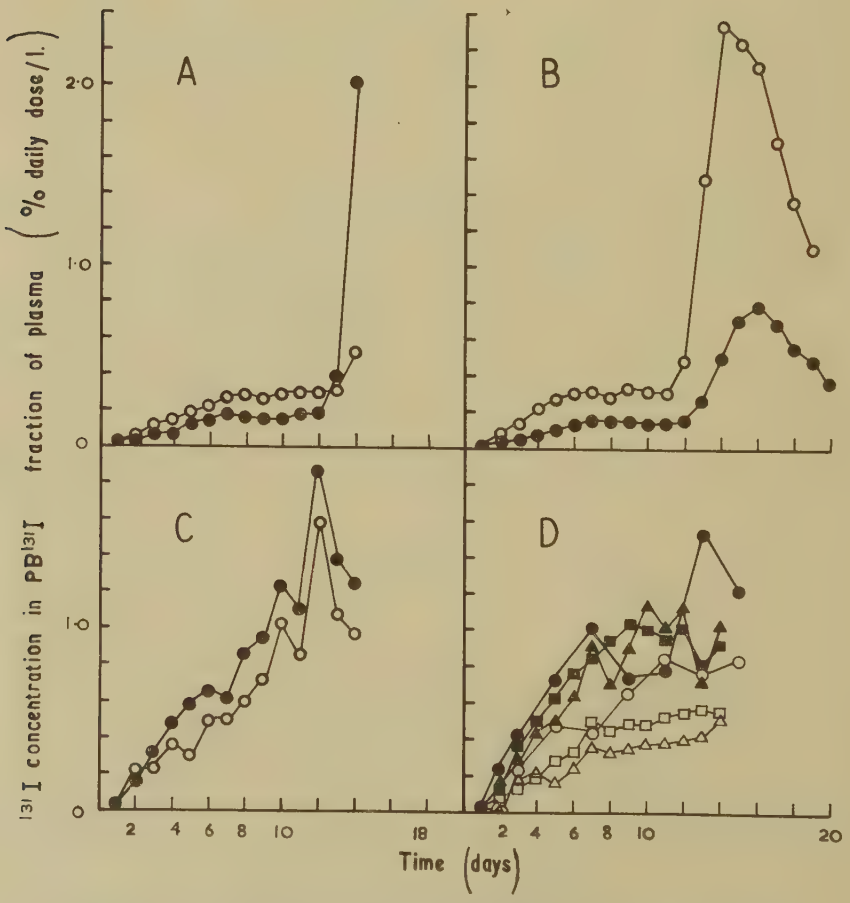
Counting methods. Five ml. samples of plasma or dialysed plasma were counted using an Ekco Type N550A scintillation head with an NaI (Tl) well-type crystal. Counts were corrected to the time of sampling. The counting efficiency for ^{131}I was 34 per cent.

Thyroid monitoring. In order to estimate the capacity of the thyroid gland to take up and to store iodine, the thyroid gland region was monitored about 2 months after dosing following a further test dose of 1 mc. ^{131}I. Control animals were examined at the same time.

In the early stages of the work thyroid monitoring was carried out using two B12 probe units (20th Century Electronics Ltd.) mounted in parallel in a suitable frame. The frame was positioned on the neck to give a maximum reading on a count ratemeter. This method measured ^{131}I present in both the thyroid gland and in the surrounding soft tissues. Later an Ekco Type N559E scintillation head with a Type 556A NaI (Tl) crystal fitted with a simple, cylindrical, lead collimator was used. The

scintillation head was built into a yoke which allowed the crystal to be positioned 4 in. from the skin. Counts were made over the thyroid region and at the base of the neck: the difference between these counts was taken as a measurement of the amount of ^{131}I in the thyroid gland. Thyroid monitoring was performed 5, 10, 15, 25 and 35 hr. after the test dose and then daily at 10.00 hr. for 3 weeks. The equipment was calibrated by counting thyroid glands in vivo in four animals in both the manners described immediately before these cows were slaughtered, and then removing the glands and counting them in the well-type scintillation counter.

Text Fig. 1.



Changes in concentration of the non-dialysable fraction of ^{131}I (PB ^{131}I) in plasma with time. A; O, Mayflower; ●, Tinkerbell (estimated thyroid radiation dose 290,000 rad): B; O, Nanette; ●, Ornament (thyroid dose 150,000 rad): C; O, Goldfinch; ●, Rosemary III (thyroid dose 70,000 rad): D; O, Nora III; ●, Colleen III; △, Jo; ▲, Gwen; □, Colleen II; ■, Nora III (thyroid dose 30,000 rad). ^{131}I was administered on days 0-13 inclusive.

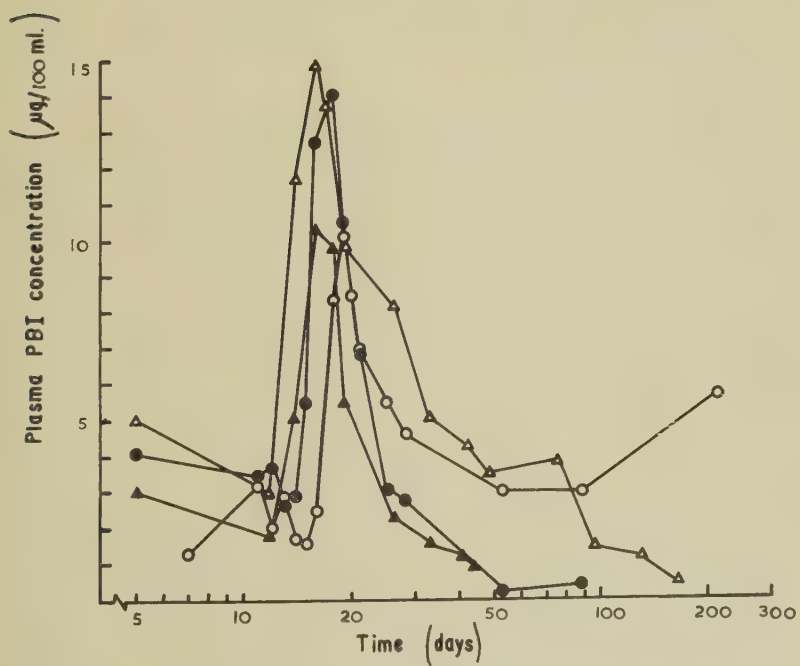
RESULTS

Early Changes

Clinical observations. In all four animals receiving 10 mc.^{131}I daily, oedema developed in the region of the thyroid gland 11 days after feeding was initiated. This swelling was transient and had disappeared completely in another 5 days. Within 1 to 2 weeks of withdrawal of ^{131}I the animals became lethargic and apathetic, rising only to feed. Except in one animal (Ornament), the milk yield was not initially affected (but see below). The subsequent histories of these animals are given later. A slight submandibular swelling lasting 1 to 2 days was seen in Goldfinch and Rosemary III, both of which were receiving a daily dose of 5 mc. and in which the thyroid radiation dose was estimated to total 70,000 rad; otherwise these and all other animals remained apparently normal.

Biochemical and haematological changes. No significant changes were observed at any time in the formed constituents or physical

Text Fig. 2.



Changes in concentration of plasma protein-bound iodine (PBI) with time. O, Mayflower; ●, Tinkerbell; △; Nanette; ▲, Ornament. ^{131}I was administered on days 0-13 inclusive.

properties of the blood, or in any of the normal chemical constituents determined other than the plasma protein-bound iodine.

In all animals which had received 10 mc.^{131}I daily, there was a sudden and marked increase in the non-dialysable (protein-bound) fraction of ^{131}I (PB^{131}I) in the plasma 12 to 14 days after dosing began (Text Fig. 1). At the same time or a few days later there was a considerable increase in the protein-bound stable iodine (PBI) in the plasma. The PBI concentration ultimately fell to below pre-dosing levels in 3 of the 4 animals at this dosage level (Text Fig. 2). A smaller release of PB^{131}I from the thyroid gland was also seen in the two animals which received an estimated thyroid radiation dose of 70,000 rad, and in one animal (Colleen III) which received a thyroid dose of 30,000 rad (Text Fig. 1). In these and all other animals there were no significant changes in PBI as compared with controls.

Thyroid function from monitoring after test doses of ^{131}I. The percentage retention of ^{131}I in the thyroid gland can be described by the expression:

$$R = K(e^{-\lambda_1 t} - e^{-\lambda_2 t})$$

where K is a constant for a given animal and describes the percentage uptake, λ_1 is the effective rate constant for elimination of ^{131}I from the thyroid gland, and λ_2 is the constant describing the rate of uptake. Values for these parameters, determined graphically, are given in Table 2.

Pathology. The animals sacrificed approximately 2 months after ^{131}I administration are listed in Table 3. No pathological changes were found in any organs or tissues other than in the thyroid gland of the cow Ornament. Microscopically, almost no functional thyroid tissue remained. Extensive necrosis of the epithelial elements was most pronounced in the central portion of the lateral lobes where persisting connective tissue stroma was the only evidence of the normal follicular pattern (Plate I, Fig. 1). As seen in sections prepared with Crossman's triple stain, the contents of the necrotic follicles consisted of granular debris and tangled strands resembling fibrin. An occasional multinucleated giant cell was seen among the disrupted epithelium of less severely affected, partially collapsed follicles near the periphery of the gland. The ultimobranchial residues at the hilus of the lateral lobe appeared to be spared. Some portions of the gland were lightly infiltrated with neutrophils, but conspicuous inflammatory changes were absent. The periphery of the thyroid consisted of dense scar tissue in which a few scattered groups of microfollicles and many small isolated aggregates of epithelial cells persisted. The collagenous tissue extended centrally as well as into the contiguous adipose tissue. The intima of many small arteries was thickened and hyalinized; in some vessels the lumen was completely obliterated. Intimal proliferation, sometimes accompanied by vacuolation and partial disruption of the

TABLE 2

Constants in the equation, $R = K(e^{-\lambda_1 t} - e^{-\lambda_2 t})$, describing retention of ^{131}I in the thyroid gland

Animal	*Estimated thyroid radiation dose (rad)*	*K* (%)	λ_1 ($hr.^{-1}$)	λ_2 ($hr.^{-1}$)
	Group 1.* Experimental animals.			
Nanette	150,000	2·4	0·030	0·053
Meta ..	15,000	13·6	0·0065	0·029
Wakeful ..		17·6	0·0060	0·034
Sadie ..	1,500	29·4	0·0052	0·031
	Control animals			
Nonesuch ..	—	31·5	0·0054	0·035
Mirth ..	—	14·9	0·0044	0·039
	Group 2.* Experimental animals.			
Mayflower ..	290,000	13·7	0·016	0·069
Tinkerbell ..		1·6	0·024	0·103
Colleen II ..	30,000	37·4	0·0067	0·048
Butterfly ..		58·9	0·0086	0·042
	Control animals.			
Nora II ..	—	47·7	0·0053	0·043
Velvet ..	—	58·6	0·0056	0·032
Elsie	—	34·7	0·0052	0·048
Rosemary II ..	—	42·6	0·0062	0·045
	Group 3.‡ Experimental animals.			
Goldfinch ..	70,000	7·0	0·0044	0·035
Rosemary III ..		6·3	0·0052	0·028
Jo	30,000	6·4	0·0044	0·028
Gwen		8·4	0·0048	0·033
Lavinia ..	7,000	6·6	0·0046	0·033
Swanny ..		7·3	0·0049	0·026
	Control animals.			
Velvet ..	—	6·6	0·0044	0·028
Lovely III ..	—	4·6	0·0048	0·027
Viola	—	5·3	0·0048	0·041
Rosemary II ..	—	8·5	0·0049	0·026
	Group 4.‡ Experimental animals.			
Nora III ..	30,000	5·8	0·0042	0·038
Colleen III ..		8·5	0·0042	0·021
Lupin	10,000	8·3	0·0054	0·029
Russet ..		8·9	0·0047	0·023
Lovely V ..	3,000	6·8	0·0044	0·023
Bunty		8·5	0·0041	0·041
	Control animals.			
Brenda ..	—	7·7	0·0040	0·026
Daffodil ..	—	5·8	0·0051	0·026

* Monitoring carried out using B12 counters.

‡ Monitoring carried out using scintillation counter.

media, was observed in the larger arteries at the hilus. Numerous macrophages containing abundant iron pigment were present in the scar tissue, where they frequently were concentrated around damaged arteries. The microscopical appearance of the thyroid glands is summarized in Table 3.

Subsequent Histories

The milk yield in all surviving animals which had received a total of 140 mc. ^{131}I was considerably lower and the time of lactation shorter than for the last lactation period before administration of the isotope (Table 4). In no other animals was the milk yield or lactation period affected.

TABLE 3

MICROSCOPICAL CHANGES IN THE THYROID GLAND OF ANIMALS SLAUGHTERED 2 MONTHS AFTER DOSING WITH ^{131}I

Animal	*Estimated thyroid radiation dose (rad)*	*Microscopical appearance*
Ornament	150,000	Essentially no functional thyroid tissue (see details in text).
Rosemary III ..	70,000	Follicles slightly hyperplastic, but probably within normal range of variation.
Gwen, Butterfly	30,000	Normal follicles filled with deeply eosinophilic colloid.
Lovely V	3,000	do.
Sadie..	1,500	do.

All surviving animals were inseminated on coming into oestrus for the first time after dosing with ^{131}I. The fertility record has been good. Two animals only have not conceived. Not all animals have yet calved: the calving history so far is given in Table 5. There have been three difficult calvings to date, two in the 10 mc. group, described in detail below, and one in the 1 mc. group. In the latter case the animal (Meta) had a prolapsed uterus and had to be slaughtered. Four calves have been born dead or have died within a few days of birth (Table 5). Of the calves born to Swanny, the one dead at birth was apparently normal, its twin died at 3 days old after scouring badly. The only animals in which changes attributable to the effects of ^{131}I were observed are those which received a total of 140 mc. of the isotope.

Tinkerbell. This animal received 140 mc. ^{131}I in January, 1959. Signs of thyroid insufficiency became apparent within 2 weeks of dosing, the animal spending most of its time in a recumbent position, rising only to feed. Although apathetic and lethargic, there was no loss of appetite and the pulse rate and respiration rate were not noticeably diminished.

TABLE 4

LACTATIONS OF COWS WHICH RECEIVED A TOTAL OF 140 MC. ^{131}I; 1, FOR LACTATION PERIOD BEFORE ^{131}I ADMINISTRATION; 2, FOR LACTATION PERIOD DURING WHICH ^{131}I WAS GIVEN; 3, FOR LACTATION PERIOD SUBSEQUENT TO ^{131}I ADMINISTRATION

		Lactation period (days)	*Total milk yield (lb.)*
Tinkerbell	1.	279	5577
	2.	125	4272
	3.	77	1061
Mayflower	1.	393	8147
	2.	135	3740
	3.	203	4028
Nanette	1.	305	6791
	2.	126	2091
	3.	see text	

TABLE 5

CALVING HISTORIES

Animal	*Predicted calving date*	*Actual calving date*	*Sex and fate of calves*
Mayflower ..	30.11.59	4.12.59	F (surviving)
Tinkerbell ..	8.12.59	12.12.59	M (born dead), F (surviving)
Nanette	5.9.59	23.9.59	M (slaughtered, normal)
Goldfinch ..	11.4.60	10.4.60	M (slaughtered, normal)
Nora III ..	16.1.61		
Colleen III ..	16.1.61		
Jo	16.6.60	26.6.60	M (d. 4 days, not examined)
Colleen II ..	21.1.60	8.2.60	F (surviving)
Meta	25.11.59	6.11.59	F (surviving)
Wakeful	Unsuccessfully inseminated 6 times		
Lupin	5.1.61		
Russet	Not inseminated at time of writing		
Lavinia	15.10.60		
Swanny	7.5.60	14.5.60	F (born dead), F (d. 3 days, scours)
Bunty	14.1.61		
Verity	7.11.59	21.11.59	M (slaughtered, normal)
Elspeth	7.1.60	26.1.60	F (surviving)
Madeline ..	30.11.59	7.12.59	F (surviving)

Within 1 month the hair became progressively dry and brittle: there was no discolouration. The animal appeared to have aged considerably within a few weeks. The skin became thick, dry and scurfy particularly over the neck where rubbed by the securing chain. The legs became swollen and, although the swelling disappeared during the succeeding 4 months, first from the fore and then from the hind legs, the joints remained permanently enlarged. The first insemination was successful. During pregnancy the animal put on flesh. About one month before parturition lameness became acute, but was partially cured by trimming the feet and dressing them

with streptomycin. Twin calves were born, a bull and a heifer. The bull calf was dead at birth but with no apparent abnormality to account for death. The heifer was apparently normal and is still surviving. Calving was difficult and there was a partial prolapse of the vagina. Despite correction both the vagina and rectum continued to tend to prolapse when the animal was recumbent. The animal's condition after calving was poor. Apathy and lethargy became more pronounced, although there was evident interest in the calf. The appetite declined progressively and the animal became severely constipated. Following a severe rectal prolapse the animal was slaughtered, 12 weeks after calving and 15 months after dosing. At this time the animal was not lactating.

Post-mortem examination revealed no abnormalities apart from bruising of the rectum, and almost complete disappearance of the thyroid gland. The organ consisted of a few tiny nodules embedded in a thin band of fibrous connective tissue. Microscopically, the remnant of the gland consisted principally of dense collagenous tissue in which only a few scattered groups of microfollicles and small isolated clumps of epithelial cells were present (Plate I, Fig. 2). The intima of many arteries was considerably thickened and often hyalinized (Plate I, Fig. 3). Focal intimal proliferation, sometimes accompanied by fragmentation of the internal elastic lamina, was seen in the larger arteries. A few macrophages containing iron pigment were scattered in the fibrous tissue.

Mayflower. This animal also showed the early swelling in the thyroid region with subsequent lethargy and apathy which was characteristic of all the cows receiving 140 mc. ^{131}I. The coat remained in good condition and there was a considerable increase in the animal's size, apparently from flesh rather than fat. Both pregnancy and calving were uneventful. The heifer calf was normal in all respects and is still surviving. The cow still appears to be in full health. Thyroid monitoring after a test dose of ^{131}I given 16 months after the period of ^{131}I administration indicated that, although the gland was capable of taking up a normal amount of the isotope, there was a much more rapid loss from the gland than in control animals. Retention at this time could be described by the equation:

$$R = 6{\cdot}1\,(e^{-0{\cdot}0239t} - e^{-0{\cdot}0433t})$$

The constants should be compared with those for the control animals in Group 4 of Table 2.

Ornament. This animal was particularly severely affected. In addition to the signs described above, within 2 weeks after dosing there was acute lameness, a rapid deterioration in condition and a marked decline in the milk yield. Sores developed, first on the bony prominences, and then spread over the back: they showed no sign of healing. The coat became very rough, but not discoloured. The animal was slaughtered 30 days after dosing ceased. At necropsy no abnormalities were found except in the thyroid gland (see above).

Nanette. This animal again showed all the initial changes associated with thyroid insufficiency. Pregnancy was uneventful. Calving was difficult. The animal went down and, despite repeated efforts to get her to her feet, remained recumbent. Although unable to rise, the animal was lively and took an interest in her calf. She was slaughtered 8 days after calving and 15 months after dosing.

The carcass generally appeared watery and contained little fat. Extensive areas of haemorrhage and degeneration, presumably of traumatic origin, were present in the medial muscle mass of the hind limbs. The thoracic and abdominal viscera appeared normal. Although readily distinguishable anatomically, the thyroid gland was merely a pale, atrophic remnant. Microscopically, it consisted almost entirely of dense fibrous tissue (Plate I, Fig. 4). Numerous small islands of microfollicles containing little or no colloid, and linear aggregates of residual epithelial cells lacking a follicular pattern were scattered throughout the dense collagenous tissue. Squamous metaplasia was observed in the epithelium of the ultimobranchial body, which otherwise appeared unchanged. Some islands of surviving follicular cells were lightly infiltrated with lymphocytes. Slight intimal thickening was present in several of the larger arteries; walls of a few large veins were disrupted by the dense scarring. Numerous macrophages heavily laden with golden brown pigment (Crossman's triple stain) were present throughout the more peripheral portions of the fibrous remnant. Some of this pigment in macrophages and in isolated clumps of epithelial cells gave a positive reaction for iron with Perls' acid ferrocyanide method.

The calf, a male, showed some oedema of the muzzle but this was attributed to trauma during calving. The oedema rapidly disappeared and, when 1 day old, the calf was standing, although a little weak, and feeding well. It was slaughtered when 5 days old and was normal in every respect. The thyroid gland was normal microscopically.

DISCUSSION

It is apparent that severe thyroid damage may occur following ingestion of a total of 140 mc. ^{131}I, i.e. sufficient to deliver to the thyroid gland a radiation dose of 150,000 to 290,000 rad. There can be little doubt that in the animals Tinkerbell, Ornament and Nanette all functional thyroid tissue was effectively, and probably permanently, destroyed. From the evidence provided by plasma $PB^{131}I$ and PBI determinations, the storage capacity of the gland was affected at an early stage, before the full amount of ^{131}I had been administered and when the gland had received an estimated radiation dose of 75,000 to 150,000 rad. Two months after dosing the ability both to concentrate ^{131}I from the blood and, presumably, to synthesize thyroid hormones had been reduced. In Tinkerbell and Nanette it is believed that ^{131}I detected in the thyroid region at this time was largely present in soft tissues other than the thyroid gland. In Mayflower, it is apparent that some tissue capable of concentrating ^{131}I from the blood survived although the storage capacity of the gland was negligible even 16 months after dosing.

The thyroid gland of the cow appears to be less sensitive to radiation than that of the sheep. In the latter species, 33,000 to 75,000 rad are sufficient to bring about extensive necrosis of the gland (Bustad *et al.*, 1955). A total radiation dose of 70,000 rad to the cow's thyroid gland appears to have a slight and transient effect not detectable by thyroid function tests or histological examination two months later. No changes were observed in the

plasma calcium or inorganic phosphorus concentration indicating that parathyroid function was not significantly affected neither were changes observed in other tissues contiguous to the thyroid gland on post-mortem examination.

Bustad *et al.*, (1955) observed a depression in the lymphocyte count in sheep given a single oral dose of 15 mc. ^{131}I, delivering a radiation dose to the thyroid gland of 33,000 to 75,000 rad. No haematological changes were observed in the present series of animals. This is not surprising since, if it be assumed that 10 per cent. of the ingested dose were taken up initially by the soft tissues of the body, it can be calculated that the total radiation dose to the whole body from 140 mc. ^{131}I would not exceed 5 rad.

The general effects of what has been termed radio-thyroid-ecrexis (Winchester, Comar and Davis, 1949) in Tinkerbell, Ornament and Nanette are generally similar to those described by Spielman, Petersen, Fitch and Pomeroy (1945) in adult cattle following surgical removal of the thyroid gland. Spielman *et al.* (1945) recorded that manifestations of oestrus were absent in their animals: this was not so in any of the cows receiving ^{131}I. Spielman *et al.* (1945) also found that growth, initially retarded, was resumed during the late stages of pregnancy: this they attributed to the beneficial effects of the thyroid gland of the developing foetus. No increase in the PBI level in the maternal plasma was observed in the present series of animals at any time during pregnancy. As in the case of thyroidectomy, radio-thyroid-ecrexis appeared to produce no diminution in fertility and normal calves were born. Spielman, Petersen and Fitch (1944) observed that thyroidectomy caused a complete cessation of lactation in about 180 days. In the series reported here, the lactation period in the three surviving animals which had received 140 mc. ^{131}I during the first month of lactation was reduced to 135 days or less and the total milk yield was correspondingly depressed. In Mayflower, the animal which was least severely affected in other respects, there are signs of recovery of milk secretion. Spielman *et al.* (1944) recorded that incomplete removal of the thyroid gland produced a temporary decline in milk secretion followed by a gradual return to former levels.

From the present work it can be concluded that the minimum radiation dose needed to damage the thyroid gland sufficiently for general effects on the animal to manifest themselves is of the order of 100,000 rad. It is difficult to relate this to an equivalent amount of ingested ^{131}I in view of the effects of season of the year and of the stable iodine content of the diet upon thyroid activity. However, assuming that 20 per cent. of ingested ^{131}I was accumulated in the thyroid gland the amount which would have to be eaten over a short period to deliver a thyroid radiation dose of 100,000 rad is about 90 mc. From a practical point of view it is of interest to know how this can be related to a level of contamination on pasture grazed by a cow. Making assumptions which are considered to

represent the worst case likely to occur under conditions of British Agriculture (Garner, 1960) a cow would ingest 90 mc. ^{131}I over a 21 day period if the initial total deposition on herbage from a single incident were 240 $\mu c./m^2$. In contrast the initial total deposition which would give the maximum permissible level of ^{131}I in milk intended for human consumption under emergency conditions following a reactor accident of 0.065 $\mu c./l$ (Anon., 1959) is 0.4 $\mu c./m^2$.

CONCLUSIONS

Results are given of experiments in which ^{131}I was administered to adult, non-pregnant, lactating cows in doses of 1·4 to 140 mc. over a 14 day period. These amounts are estimated to have delivered radiation doses to the thyroid gland of 1,500 to 290,000 rad.

The clinical effects of ablation of the thyroid gland by means of radiation (radio-thyroid-ecrexis) were similar to those described after surgical removal of the gland.

The minimum radiation dose needed to have a deleterious effect on the animal is estimated to be of the order of 100,000 rad.

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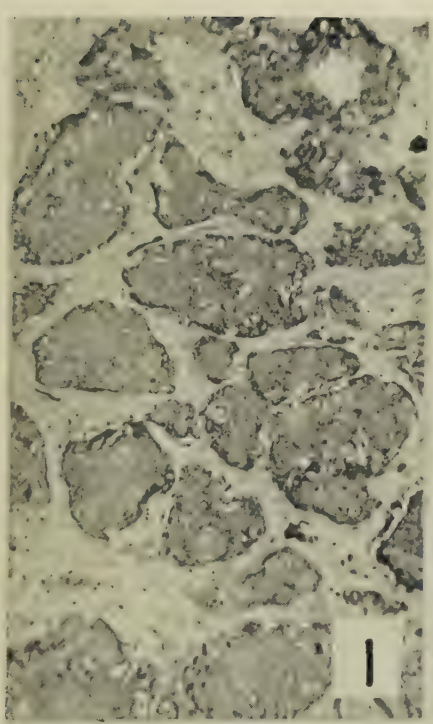
LEGENDS TO ILLUSTRATIONS

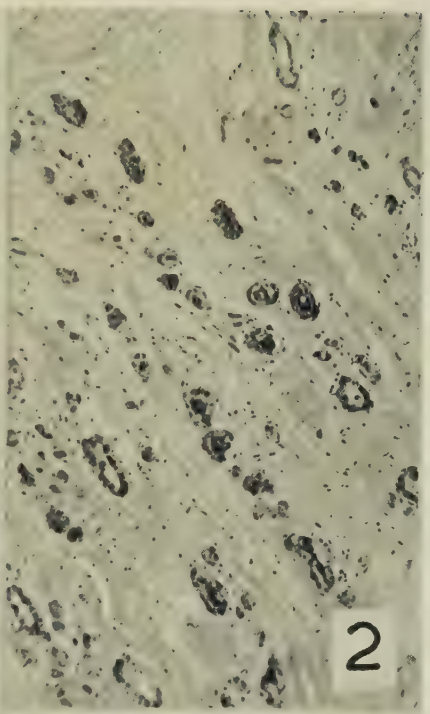
Fig. 1. Thyroid gland from Ornament. Section near edge of lateral lobe showing complete necrosis of the follicles, and variable fibrosis of the stroma. Crossman's triple stain. × 100.

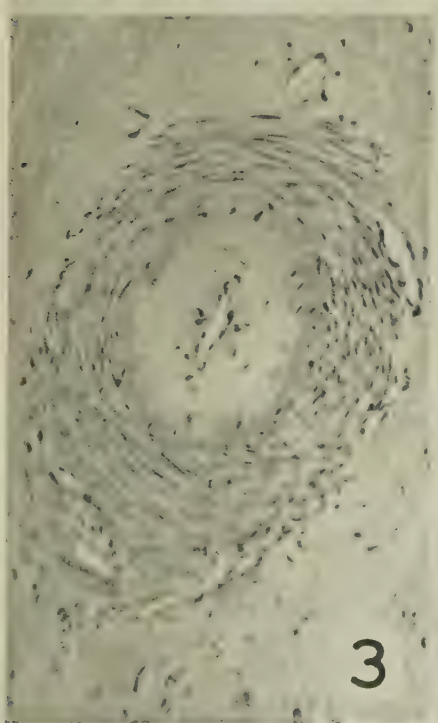
Fig. 2. Thyroid gland from Tinkerbell. Only scattered residual follicles are seen in the dense collagenous tissue. Crossman's triple stain. × 100.

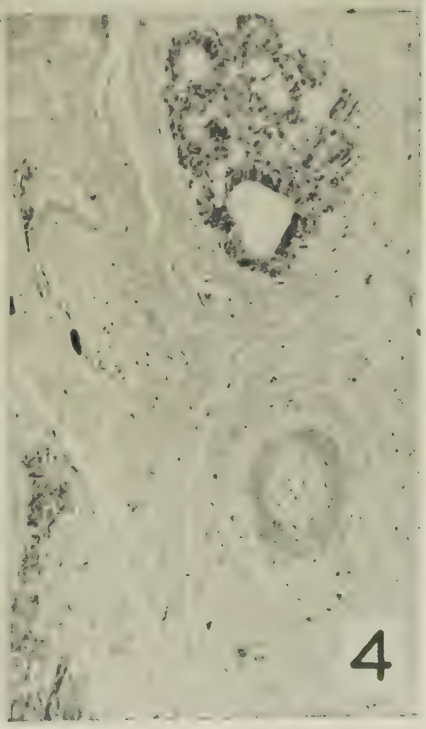
Fig. 3. Thyroid gland from Tinkerbell. Pronounced fibrosis of the intima of a small artery. H.E. × 170.

Fig. 4. Thyroid gland from Nanette. Groups of atrophic follicles are isolated in the dense collagenous tissue. The lumen of the small artery is prominently attenuated by the thickened intima. Crossman's triple stain. × 100.









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PATHOLOGICAL AND BIOCHEMICAL OBSERVATIONS ON VIRUS-INDUCED *OSTEOPETROSIS GALLINARUM*

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INTRODUCTION

The term osteopetrosis was introduced by Karshner (1926) to describe a human osteopathy characterised by a great increase in size and density of all bones save those of membranous origin. Prior to this the condition was known as "marble bones" or Albers-Shönberg disease. In man it is a rare hereditary condition, not sex-linked, but transmitted as a simple recessive to the progeny of closely related persons. There is an equal incidence in the sexes. The cause is unknown.

An analogous condition in domestic fowls was described by Pugh (1927) as a "sporadic diffuse osteo-periostitis". Early work on avian osteopathies, including osteopetrosis, has been adequately surveyed by Jungherr and Landauer (1938), who named the disease *Osteopetrosis gallinarum,* and demonstrated its transmissibility by suspensions of cells obtained from various tissues in florid cases; preparations dried in a desiccator for at least 105 days retained their infectivity. Recently Holmes (1958) succeeded in transmitting osteopetrosis originating in a hen, one of three birds affected in a flock of about 100 fowls. He used day-old chicks of mixed origin, and injected blood, marrow, or unfiltered plasma intraperitoneally. He was also able to produce the disease in young chickens which had been injected as embryos by the intra-amniotic route. Subsequently Holmes (1959) was able to show the true filterability of the agent, its probable presence in the semen of infected males and its infectivity when administered orally.

The present series of experiments were designed to investigate the susceptibility of inbred fowls to the disease, to study the manifestation of any concomitant conditions which might occur in infected birds, and to investigate the blood picture both microscopically and chemically. To the latter end, calcium (total and diffusible) and citrate levels of plasma and its acid and alkaline phosphomonoesterase activities were measured.

MATERIALS AND METHODS

Refrigerated, heparinised, whole blood from a florid case of the disease was kindly provided by Dr. J. R. Holmes of the Department of Veterinary Medicine, University of Bristol. Upon receipt it was immediately injected intraperitoneally into 24 Brown Leghorn chicks (day-old; 18♂, 6♀) from the inbred flock maintained at the Agricultural Research Council's Poultry Research Centre. Twelve 15-day embryos were also

inoculated on the chorio-allantoic membrane by Dr. J. G. Carr. The development of detectable lesions, e.g. thickening and heat in the metatarsi was checked periodically. All birds were examined radiologically at least once, irrespective of the development of visible lesions. Certain birds killed for examination and histology were also X-rayed post-mortem. Tissues removed for histology included bone, liver, spleen, gonads, thymus, thyroid and parathyroid, adrenal and kidney. These were fixed either in formol-saline for subsequent decalcification of bone, or in Susa. Paraffin and celloidin sections were stained by haematoxylin and eosin, Mallory's trichrome, or Gordon and Sweet's method for connective tissue counterstained with H. & E.

Blood Analyses

Blood was taken from the wing vein into a heparinised syringe and centrifuged at 5°.

Haematocrits were measured in Wintrobe tubes at 3,200 *g* and 5° (Bell, 1957).

Plasma citrate was measured by conversion into pentabromoacetone following exactly the procedure of Ettinger, Goldbaum and Smith (1952); the pentabromoacetone was then determined either by the method of Taussky and Shorr (1947) or by that of MacDonald and Waterbury (1959). The latter procedure was found to give, in general, more consistent results although, on the scale adopted, it required the optical measurements to be made in the micro cell (1 cm. light path) of the Unicam SP 500 spectrophotometer.

Total calcium in plasma was determined by the method of Henley and Saunders (1958). Using the same analytical procedure diffusible calcium was measured in plasma ultrafiltrates obtained by a speeded-up modification (15 to 30 mins.) of the centrifuging technique of Prasad and Flink (1957).

Red cell counts were made with a Thoma haemocytometer.

Plasma alkaline phosphatase activity was measured as milligrams of 4-nitrophenol liberated by 100 ml. of plasma from 4-nitrophenyl phosphate in 60 mins. at 37° in 0·1 molar $NaHCO_3$-Na_2CO_3 buffer (pH 10·08 at 37°) (Bell, 1960).

Acid phosphatase activity was assessed at pH 4·9 in a similar manner but using 0·2 molar citrate or acetate buffers (Bell, unpublished).

RESULTS

Five chicks died 6 to 20 days after inoculation due to accidental yolk-sac damage by the intraperitoneal injection. Of the surviving 19 birds, 12 eventually developed marked diaphyseal enlargement of the long bones of the wing, including the shoulder girdle, and leg; 10 of the 12 were males. Four of the remaining 7 showed occult lesions detected only by radiology; of these 3 were males. Two of the 7 hatched birds infected in ovo subsequently developed osteopetrosis, at 6 and 9 weeks respectively, both being males.

A number of these birds (15 to 18 weeks old) were used for biochemical estimations, and Table 1 summarises the results. The controls were of the same age.

TABLE 1

SOME CLINICAL AND BIOCHEMICAL FINDINGS IN AVIAN OSTEOPETROSIS

No.	Sex	Visible severity of lesions	Red cell volume %	Erythrocytes $\times 10^6$	Plasma analysis: Phosphatase activity against 4-nitrophenyl phosphate (See text) — Acid 15 wks.	Acid 18 wks.	Alkaline 15 wks.	Alkaline 18 wks.	Ca (mg./100 ml.) Total 15 wks.	Ca (mg./100 ml.) Diffusible 15 wks.	Citrate mg./100 ml. 15 wks.	Citrate mg./100 ml. 18 wks.
1	♀♂♂♀♂♂♀♂♂♂♂♂♂♂	0	36·0	2·832	11	14	72	66	10·9	5·2	14·9	10·8
2		0	35·5	2·684	12	11	80	130	10·4	5·8	11·1	10·4
3		0	29·0	2·512	8	7	83	120	10·6	5·2	15·8	12·0
4		0	34·0	2·895	6	7	114	160	10·5	4·4	8·1	9·2
5		+	35·5	2·504	5	10	126	160	11·0	5·6	14·7	8·8
6		++	37·0	2·965	8	11	143	380	11·0	5·4	7·4	6·3
7		0	34·0	2·210	8	10	195	320	11·8	6·0	17·0	9·2
8		+	34·0	3·240	12	10	245	320	11·4	6·0	9·0	8·1
9		++	33·5	1·552	10	12	340	210	11·4	5·8	9·8	8·9
10		+++	34·0	2·322	6	—	405	—	—	—	7·6	10·0
11		++++	34·0	2·980	17	10	550	760	11·0	6·4	10·7	11·0
12		++++	34·0	2·032	12	—	610	—	—	—	10·1	—
13		0	36·0	2·282	12	—	640	—	—	—	13·4	—
14		++++	35·5	2·340	12	12	740	820	10·2	5·2	8·0	10·0
					CONTROLS							
1	♂♀♀♂♀♂♂♂♂	—	35·5	2·900	7	7	236	110	10·0	5·6	6·7	10·0
2		—	35·0	2·680	6	5	89	83	11·7	6·0	8·4	7·7
3		—	33·0	2·360	10	7	80	77	10·6	5·6	6·7	8·8
4		—	31·0	2·580	8	8	95	120	12·2	5·6	7·3	9·0
5		—	39·0	3·460	10	10	89	100	10·8	5·6	8·2	8·3
6		—	36·0	2·850	8	9	87	74	10·5	5·8	6·6	9·0
7		—	36·0	3·264	6	6	118	140	10·8	6·0	9·3	11·0

Pathology

It is not proposed to detail the pathological process of abnormal bone production as this has already been done (Reinhardt, 1930; Jungherr and Landauer, 1938). Briefly, and in agreement with previous investigators, the tarsometatarsals show the first macroscopically visible change, characterised by thickening and increased heat of the part. Examination shows a similar change affecting the long bones of the wing and leg, but not the digits. The birds are somewhat stunted and the secondary sex characteristics are retarded. The periosteum is thickened; the bone is most swollen at the mid diaphysis, the articular extremities are virtually uninvolved. This results in a fusiform swelling of the bone. Commonly, the initial lesion detectable by radiology is an asymmetrical endosteal proliferation, and this is followed by periosteal osteogenesis in definite layers. Eventually the medullary cavity may be completely obliterated (Fig. 1), while some evidence of patchy bone resorption may be seen, suggesting a belated, and possibly osteoclastic, attempt to remodel the bone on more normal lines.

The highly vascular new bone laid down forms trabeculae converging centripetally towards the centre of the shaft (Fig. 2). Large distorted Haversian canals contain vessels, along with a scanty fibrous matrix probably derived from modified osteoblasts (Pepler, 1960). No haemopoietic tissue can be found, and the cavities are palisaded with osteoblasts, while osteoclasts are absent or rare except where resorption is proceeding (Fig. 3). A mosaic of cement lines, indicating new bone formation, is characteristically present between the abnormal interstitial and normal lamellar bone (Fig. 4).

The virtual obliteration of the long bone marrow is to some extent compensated for by haemopoietic tissue in those bones which are not commonly involved, namely the ribs and one or two other minor sites. It seems improbable that these sources are in themselves sufficient to maintain normal numbers of blood cells; the RBC counts are not affected, but blood films show many immature red cells and a deficiency of white cells typical of a myelophthisic anaemia. If a heparinised sample is centrifuged and a smear from the pinkish buffy coat (1 to 1·5 per cent. of the PCV), is examined, some immature white cells are to be seen together with many lymphocytes, but the majority of the cells are erythroblasts.

Myelopoiesis is a common feature in the livers and kidneys. In the latter it can be very marked in the interstitial tissue. An interesting feature is a progressive "cirrhotic" change in the livers of some females, especially in those showing minor bone lesions (Fig. 5). This is characterised by a peculiar endotheliosis due to hyperplasia of the sinusoidal reticulin fibrils and dilatation of the sinusoids, which show precipitated material in the space of Dissé, presenting a picture not unlike the oedema of serous hepatitis. The liver capsule is markedly thickened. Such cases usually have an enlarged congested heart, with an engorged right atrium.

The spleen in both sexes is almost invariably atrophic and shows histologically, a marked depletion of the germinal lymphoid centres. This finding is the reverse of that reported by Jungherr and Landauer (1938). Small hyaline patches not obviously associated with any structure are a common feature.

The gonads may show retarded development, even in birds aged 6 months or more. The adrenals tend to be slightly enlarged, but retain the normal distribution and proportions between cortical and medullary tissue. The thyroids appear normal, as do in general the parathyroids. The latter, exceptionally, may show a mild hyperplasia, or even as in one instance, a marked fibrosis and cystic metaplasia involving one gland, while another from the opposite side of the neck showed a clear cell (wasserhelle) hyperplasia (Figs. 6 and 7). The thymus appears normal.

In the cases which have come to post-mortem examination to date there has been no associated leucosis or any other neoplastic change either in bone or in other organs. No evidence of nerve involvement (neurolymphomatosis) was seen.

DISCUSSION

The blood picture of fowls with this virus-induced osteopetrosis is at no time characteristic of a leukaemia. The erythrocytes show the considerable poikilocytosis, anisocytosis and basophilia characteristic of a myelophthisic anaemia, but are not reduced in number. A small number of erythroblasts are present. In view of the almost complete obliteration of the major myelopoietic centres, the compensatory extramedullary myelopoiesis is surprisingly efficient in supporting a reasonable degree of superficial health in these osteopetrotic birds.

In advanced cases the only invariable visceral abnormality is shown by the spleen. This organ resembles the so-called senile atrophic spleen in man which is considered to arise as a result of a deficient blood supply. However, hyaline changes of the splenic vessel walls are not present, although hyalinization occurs elsewhere in the organ. In addition there appears to be a disposition towards fibrosis in the livers of females; this sex would seem to be less susceptible to the more florid manifestations of osteopetrosis. None the less in these females also, the medullary cavities of the long bones are largely effaced by inward growth of the endosteal wall. The gonads, at 15 to 18 weeks of age, are undeveloped. Whether this may indicate some suppression of the gonadotrophic activity of the anterior pituitary in osteopetrosis could be shown only by study of the anterior pituitary. The oestrogen-androgen balance, as assessed by the method of Campbell (1957, 1960) showed considerable variation between individual birds regardless of sex but this is a usual finding in birds of the same age-group (Campbell, 1960). No data regarding oestrogen-androgen balance have so far been obtained from osteopetrotic females (15 to 18 weeks) with damaged livers.

The recent publication of Taylor, Moore and Hertelendy (1960) is of interest. These authors have found that the bones of the domestic fowl may be divided into two categories according to their citrate content. Wing and leg bones, coracoid and scapula are relatively low in citrate while the ribs, sternum, pelvic girdle, digits and skull are relatively high in that anion. In the earlier stages, at least, osteopetrosis affects only those bones classified by Taylor *et al.* (1960) as low-citrate ones. They found also in 5 instances that the cervical vertebrae contained citrate at the "high" level in 2 birds while, in the remaining 3, the vertebrae belonged to the "low" citrate class. So far we have not seen any evidence of osteopetrosis of the cervical vertebrae but are paying attention to this problem as our experimental birds get older. We have not as yet examined the bone citrate of osteopetrotic birds.

The results of the biochemical measurements on the plasma are on the whole consistent with very active bone mineralisation. Plasma alkaline phosphatase activity, assayed against 4-nitrophenyl phosphate, is at a greatly enhanced level, especially in those cases which show patent lesions, and is raised well above that of the controls in those birds having lesions detectable only by radiography. There is considerable evidence that plasma alkaline phosphatase originates largely in osteoblasts (Hall and King, 1930; Changus, 1957; Henrichsen, 1958; Burstone, 1959) which are prominent in osteopetrotic bone. Siller (1959) reports a high level in a hen with osteogenic sarcoma where there is, of course, great osteoblastic activity. Furthermore, the fibrous stroma in the enlarged blood spaces of the affected bone is also probably composed of altered osteoblasts; such fibrous dysplasic tissue has been shown to be histochemically similar to osteoblasts by Changus (1957).

The plasma acid phosphatase activity (against 4-nitrophenyl phosphate) averages a little higher than in the controls, but we do not wish to stress this point as it is not yet known what interpretation is to be placed on differences at these levels. In cases of "cage layer fatigue" (Bell, Siller and Campbell, 1959) among laying pullets, which involves severe bone resorption with marked osteoclasis, elevated plasma acid phosphatase levels have been noted (unpublished); the levels found in osteopetrotic birds may reflect a lack of osteoclastic activity. It is known that osteoclasts contain acid phosphatase (Changus, 1957; Burstone, 1959; Pepler, 1960) and some of the plasma enzyme may therefore originate in these cells, thus parallelling the osteoblastic source of plasma alkaline phosphatase.

Plasma citrate levels in the osteopetrotic birds covered a moderate range of values, especially at 15 weeks, where more than half showed levels higher than those of any of the controls. At 18 weeks this difference was less apparent. The values for the controls closely resembled those found by Taylor *et al.* (1960) for birds of the same age group. In mammals an elevated plasma citrate may be cor-

related with increased activity of the parathyroid (Neuman, Firschein, Chen, Mulryan and DiStefano, 1956; Firschein, Martin, Mulryan, Strates and Neuman, 1958), but in the osteopetrotic birds it cannot be stated that this gland showed clear evidence of hyperactivity except, possibly, in the case of No. 11 (male) where one gland was fibrotic and cystic and presumably inactive, while another from the opposite aspect of the neck showed a clear-cell hyperplasia. We interpret this, not as a primary parathyroid lesion, but rather as a reflection of a severely abnormal bone metabolism. It is not known whether increased parathyroid activity in birds is accompanied by elevated plasma citrate.

Both the diffusible and non-diffusible fractions of plasma calcium were in the normal range. This is, perhaps, not surprising since the birds were fed on a well balanced diet and the absence of renal lesions shows competence to maintain a normal physiological level of diffusible calcium, since Fussell (1960) has recently demonstrated that the kidney of the fowl plays a major role in calcium excretion. The normal level of the non-diffusible calcium fraction is indicative of the absence of any state of oestrogenisation which would lead to lipoproteinaemia with increased binding of calcium to large molecules.

It may be recalled that Burmester and Cottral (1947) reported that inoculation of fowls with RPL/12 material induced not only lymphoid tumours but also a proportion of cases of osteopetrosis. More recently, Lesher and Burmester (1955) found that in birds bearing the RPL/12 tumour, plasma alkaline phosphatase activity was at a much lower level than in their controls. No difference was found in the two levels of acid phosphatase activity.

As no reports of osteopetrosis accompanying RPL/12 have appeared in recent years and since Lesher and Burmester (1955) detected a fall in plasma alkaline phosphatase activity in birds bearing the RPL/12 tumour, it seems distinctly possible that the original RPL/12 may have been contaminated by an osteopetrotic virus, or that RPL/12 may have mutated with the passage of time, or that a latent osteopetrotic virus was unmasked in those birds used in the earlier experiments. In our present cases of osteopetrosis no evidence of lymphoid or other tumours associated with the so-called "leucotic complex" have come to light. Holmes (personal communication) deduces, as a result of his recent investigations, that there is strong evidence that osteopetros should be considered as a separate entity in avian pathology and that it therefore does not come within this complex of neoplasms of haemopoietic tissues.

CONCLUSIONS

Eighteen cases of osteopetrosis induced by a field virus provided by Holmes (1959) have been examined clinically, biochemically and post-mortem. Infection of day-old chicks resulted in a high propor-

tion of cases showing well advanced lesions at fourteen weeks. Males were more susceptible than females.

Bone lesions were, at least initially, confined to the long bones of the wing and leg. Subsequent involvement of the pectoral girdle occurred.

No changes in the viscera could be detected beyond splenic atrophy, delayed maturation of the gonads, and in some females which showed minor bone involvement, a peculiar endotheliosis of the liver, grossly simulating cirrhosis. In the main, the parathyroids appeared normal.

The blood picture was characteristic of a myelophthisic anaemia. No evidence of a concomitant leukaemia was found in the osteopetrotic birds. The lack of marrow in the long bones was compensated by development of extramedullary erythropoietic centres.

The plasma alkaline phosphatase activity was high in those birds which showed definite clinical or radiological signs; this is indicative of the high osteoblastic activity in the bone. Although values for acid phosphatase averaged a little higher than in the controls it was not possible to correlate this with increased osteosclasis.

Calcium, both diffusible and non-diffusible, appeared unaffected by the dystrophy. Plasma citrate covered a moderate range of values, but averaged slightly higher than was found in the controls.

No instance of neoplasia has been detected in any of our cases to date.

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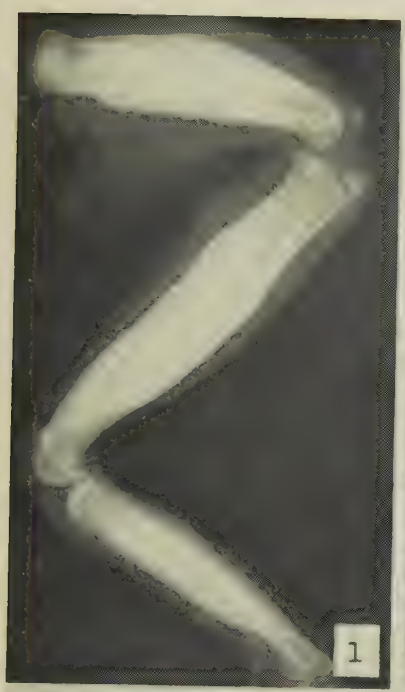

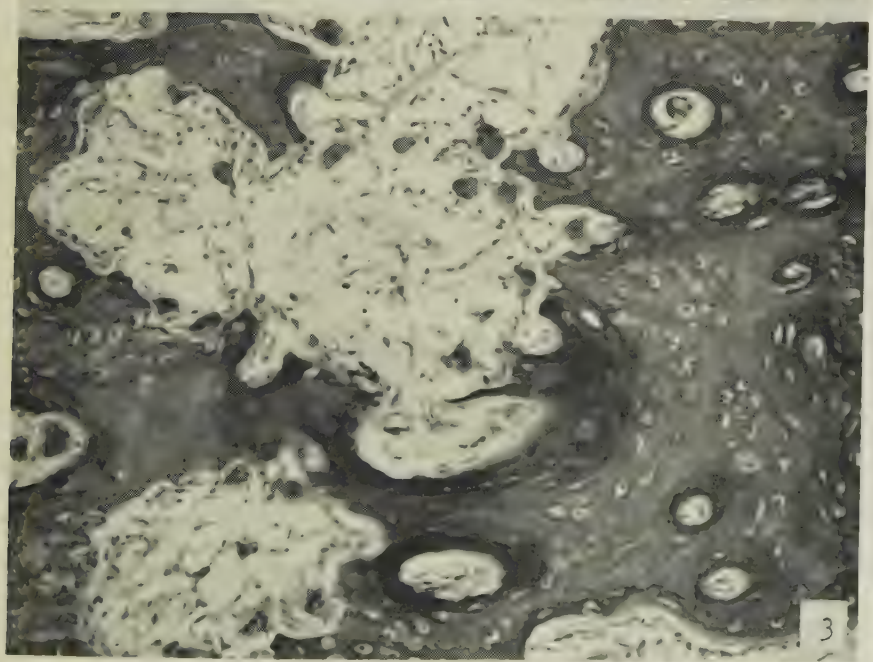

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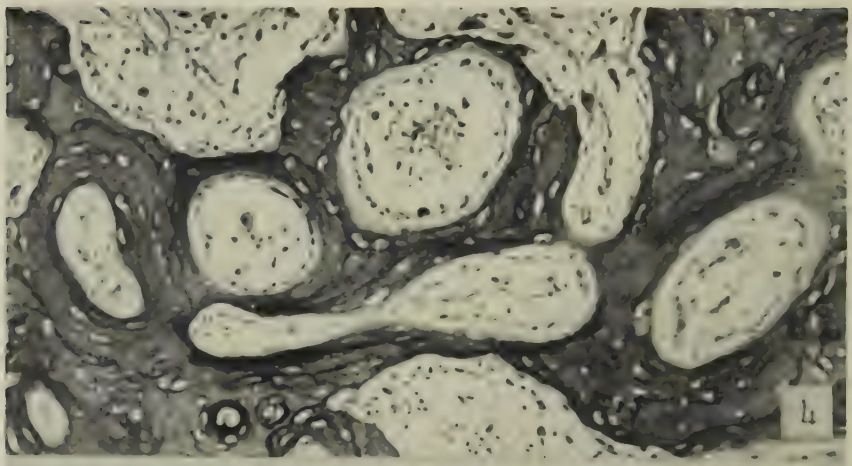
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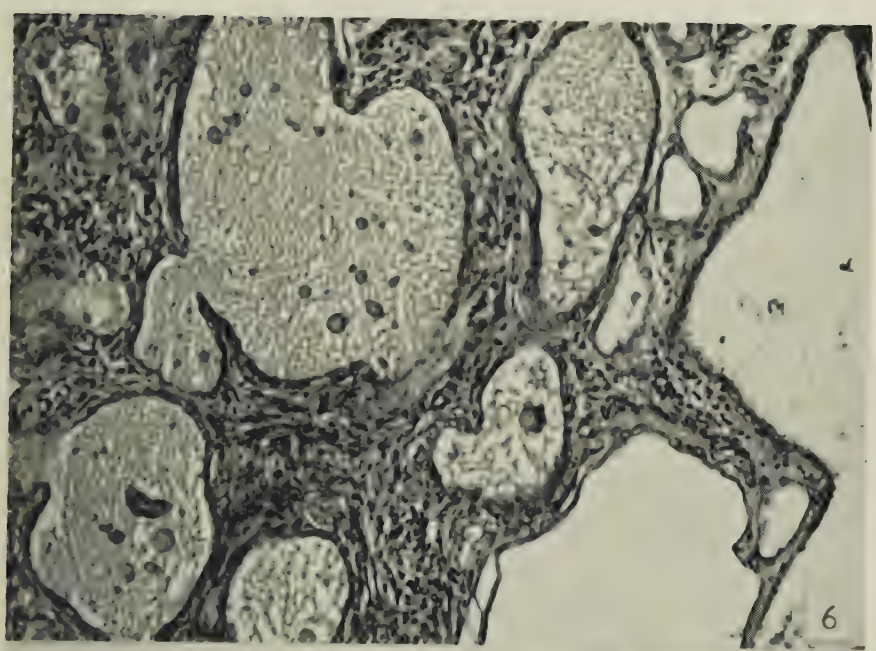
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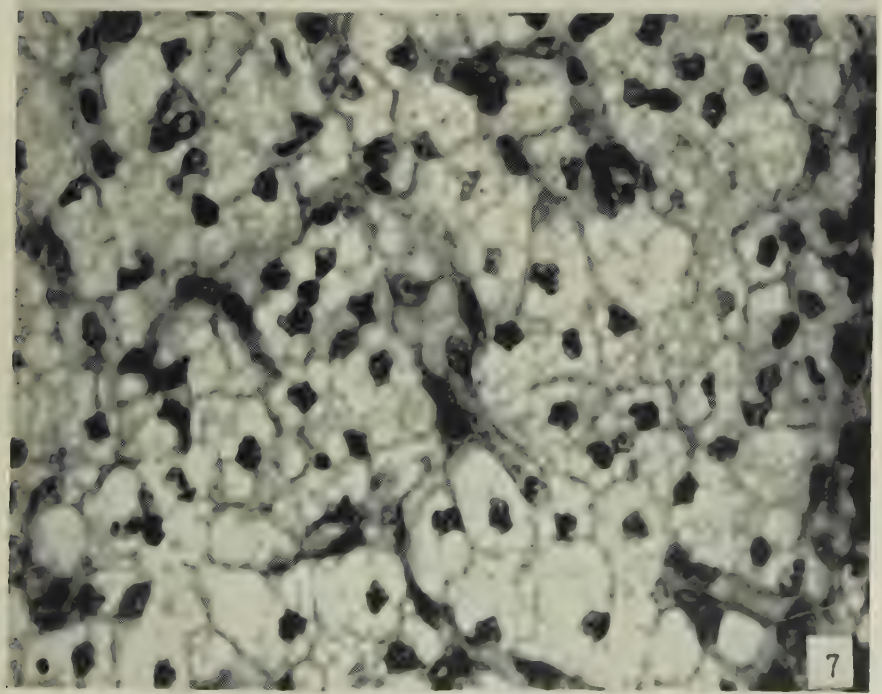
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LEGENDS TO ILLUSTRATIONS

Fig. 1. Radiograph of leg bones from an induced case of osteopetrosis in a female, showing obliteration of medullary cavity, and active bone resorption. The orginal shows lamination of the periosteal surfaces, suggestive of periodic bone-forming activity. × ⅔rds.

Fig. 2. T.S. tibia, showing centripetal orientation of grossly distorted Haversian canals. H & E × 40.

Fig. 3. T.S. tibia. Note osteoclastic activity and formation of associated lacunae. H & E. × 180.

Fig. 4. T.S. Femur. Fibrosis of marrow, palisading of osteoblasts, and the mosaic of new bone formation are all evident. There are few osteoclasts. This is from the same case as Fig. 3 and shows that bone formation and bone resorption proceed independently at different sites. H. & E. × 180.

Fig. 5. Liver lesion in a female. Note capsular fibrosis and contraction, also engorged right atrium. × ⅔rds.

Fig. 6. Fibrosis and cystic metaplasia, parathyroid. H. & E. × 180.

Fig. 7. Clear cell ("wasserhelle") hyperplasia, parathyroid. Same case as fig. 6. H & E. × 660.

FURTHER OBSERVATIONS ON THE PATHOGENICITY OF *PASTEURELLA HAEMOLYTICA* FOR YOUNG LAMBS

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INTRODUCTION

It was reported by Smith (1959, 1960b) that strains of *Past. haemolytica* isolated from sheep could be classified into two Types, designated A and T. Cases of enzootic pneumonia (Montgomerie, Bosworth and Glover, 1938) yielded A strains while the septicaemic disease of older lambs (Stamp, Watt and Thomlinson, 1955) was associated with Type T infection. Generalised Type A infections in lambs within the first few weeks of life were encountered by Smith (1960a) who demonstrated the high susceptibility of 3-week old lambs and the high resistance of young adult sheep to intraperitoneal and intravenous inoculations of a Type A strain freshly isolated from a field case; a Type T strain was of much lower virulence for young lambs, but this might have been the result of repeated laboratory subculture of the organism.

The present studies are concerned with the virulence for 3-week old lambs of Type A strains isolated from cases of enzootic pneumonia and also of a number of Type T strains.

MATERIALS AND METHODS

Strains of Past. haemolytica. To indicate its type, each strain was given a reference number preceded by the letter A or T. The results concerning strains A-158 and T-7157H are quoted from a previous publication by Smith (1960a) from which full experimental details may be obtained. Nos. A-158 and T-7157H were obtained from cases of septicaemia in a 3-week old and 6-month old lamb respectively. The 9 strains used in the present studies originated from different outbreaks of disease. Nos. A-1, A-2, A-3 and A-4 were isolated from cases of enzootic pneumonia in adult sheep. Nos. T-5, T-6a, T-7, T-8 and T-9 were isolated from cases of septicaemia in lambs over the age of 6 months. Two strains, Nos. A-1 and T-5, were preserved for several months at -50°C in pieces of diseased tissue. The remainder were kept for various periods in artificial culture and on frozen blood-agar slopes. The number of laboratory cultures undergone by each strain prior to inoculation is shown in the Table.

Experimental animals. Lambs obtained from two farms were used when between 3 and 4 weeks old. Adult sheep consisted of 1 and 2-year old animals from the Institute flock.

Inocula. These were prepared by making appropriate dilutions of 5-hour infusion broth cultures of the various strains. The dose volume per animal was 2 ml. and inocula were injected intraperitoneally.

Viable counts. The method of Miles and Misra (1938) was used to determine the viable counts of inocula and also of peritoneal exudates from lambs which died as a result of experimental infection. The average result of 3 replicate counts was in each case taken to be the true value.

RESULTS

Table 1 shows the results of experiments designed to provide information on the virulence for young lambs of a number of *Past. haemolytica* strains inoculated intraperitoneally. As stated above, the findings concerning strains A-158 and T-7157H have already been reported in a paper by Smith (1960a) and they are quoted in the Table to facilitate an over-all assessment of the information available at present. The remaining experiments using 4 Type A and 5 Type T strains were performed within a period of 10 days during the spring of 1960. All lambs which died in the course of this work did so from acute fibrinous peritonitis within 36 hours of inoculation. Peritoneal exudates varied in volume from 10 ml. to 50 ml. Immediately or shortly after death, counts of viable bacteria in these exudates were carried out and the results in each case indicated marked bacterial multiplication. *Past. haemolytica* was recovered from the lung, liver and spleen of each dead animal. Surviving lambs were afebrile and apparently healthy when examined 24 and 48 hours after inoculation.

The first two experiments were performed with strains A-1 and T-5, both of which had been subjected to the minimum number of artificial cultures between isolation and inoculation. Five-hour broth cultures of the two strains were titrated intraperitoneally in pairs of lambs using 100-fold dose dilutions and 2 ml. dose volumes. The largest dose of each strain consisted of 2 ml. of 1/10 broth culture dilution. Parallel titrations were carried out in pairs of adult sheep, each pair consisting of a 1-year old and a 2-year old animal. None of the adults inoculated died or showed any symptoms. As may be seen from the Table strain A-1 possessed considerable virulence for the lambs and gave results of the same order as those previously obtained with strain A-158. Comparable doses of strain T-5, on the other hand, produced no deaths or apparent symptoms in lambs. Survivors from both experiments were discarded 3 weeks later at which time they still appeared healthy.

At this stage of the investigation an examination of the results already obtained with strains A-1, A-158, T-5 and T-7157H suggested that young lambs might be more susceptible to experimental infection with Type A strains than Type T. With this possibility in mind 7 pairs of lambs were inoculated intraperitoneally with single dose levels of strains A-2, A-3, A-4, T-6a, T-7, T-8 and T-9 respectively. The Type A strains were inoculated in 2 ml. volumes of 1/1000 dilutions of 5-hour broth cultures and Type T inocula were prepared in the same way except that 1/100 culture dilutions were used. In this way Type A strains were administered in doses of not more than $1{\cdot}4 \times 10^6$ viable organisms and Type T strains in doses of not less than $11{\cdot}0 \times 10^6$. Numbers of subcultures undergone by the various strains between isolation and inoculation are shown in the Table. Only one of the three Type A strains failed to produce death and this strain was exceptional in that it had undergone many sub-

TABLE 1

INTRAPERITONEAL INOCULATION OF *PAST. HAEMOLYTICA* STRAINS, TYPES A AND T, INTO YOUNG LAMBS

No. of artificial cultures undergone by strains before inoculation	*Virulence of Type A strains for lambs*				*Virulence of Type T strains for lambs*			
	Strain No.	*Dose of viable organisms (millions)*	*Deaths*	*Viable bacteria in peritoneal exudates at death (millions/ml.)*	*Strain No.*	*Dose of viable organisms (millions)*	*Deaths*	*Viable bacteria in peritoneal exudates at death (millions/ml.)*
2	A-158*	58·0	2/2	18,500·0 and 45,000·0	T-5	142·0	0/2	....
		0·58	1/2	27,500·0		1·42	0/2	....
		0·0058	2/2	45,000·0 and 15,500·0		0·0142	0/2	....
		0·000058	1/2	2,350·0		0·000142	0/2	....
	A-1	114·0	2/2	17,500·0 and 26,000·0		....	....	....
		1·14	2/2	23,000·0 and 32,000·0	T-6b	0·8	0/3	....
		0.0114	1/2	2,350·0		....	....	....
		0·000114	0/2	....		....	....	....

Table 1—*Continued*

4 to 6	A-2	0·9	1/2	2,750·0	T-6a	13·0	1/2	7,500·0
	A-3	1·4	2/2	15,000·0 and 10,500·0	T-7	20·0	0/2	....
About 20		....	....	....	T-8	30·0	0/2	....
		....	....	....	T-9	11·0	0/2	....
Many	A-4	....	....	....	T-7157H*	140·0	1/2	8,500·0
		1·0	0/2	....		1·4	0/2	....
		....	....	....		0·014	0/2	....
		....	....	....		0·00014	0/2	....

* Results previously recorded by Smith (1960a).
Strain T-6a was re-numbered T-6b after passage through one lamb.

cultures since isolation. Of the four Type T strains inoculated three failed to produce death. It should be pointed out, however, that two of these strains had undergone about 20 artificial cultures prior to inoculation. Strain T-6a inoculated in doses of 13×10^6 viable organisms killed one of a pair of lambs. The death of this lamb was associated with considerable bacterial multiplication in vivo as may be seen from the high viable count present in peritoneal exudate. However the organism freshly isolated from the carcase (strain T-6b) produced no illness or deaths in a further 3 lambs inoculated with the smaller dose level of $0{\cdot}8 \times 10^6$ viable organisms. The virulence of this strain did not therefore appear to be comparable with that of the majority of Type A strains examined.

DISCUSSION

It was shown by Smith (1960a) that young lambs were highly susceptible to intraperitoneal inoculation with a Type A strain of *Past. haemolytica* freshly isolated from a 3-week old lamb which died as the result of septicaemia. From the present investigations it is now clear that comparable results may be obtained with Type A strains recently isolated from cases of enzootic pneumonia in adult sheep. Such strains inoculated intraperitoneally into 3-week old lambs were shown to be capable of high degrees of multiplication leading to death from acute fibrinous peritonitis. Adult sheep were resistant to the doses used. Under similar experimental conditions virulence for young lambs of a number of Type T strains derived from cases of septicaemia in lambs over the age of 6 months was found to be either very slight or completely lacking. This was the case notwithstanding the use of one strain freshly isolated from frozen diseased tissue and of a further two strains which had undergone only between 4 and 6 subcultures prior to inoculation. It may be pertinent to recall the account given by Smith (1960a) of field cases of septicaemia in lambs within the first few weeks of life; of 18 cases examined 17 were due to Type A infection and only one to Type T infection. A relatively small number of future experiments performed with freshly isolated strains should decide beyond doubt whether a generalisation as to the difference in virulence between Types A and T of *Past. haemolytica* for young lambs is justified. Unfortunately certain practical difficulties are inevitably associated with work of this nature. They include either the high cost of breeding lambs for experimental purposes or the inconvenience of bottle-feeding purchased lambs. In addition there is only a relatively short period in each year during which experiments can be carried out. It is felt that the present observations should be of some assistance in future studies.

CONCLUSIONS

Investigations on the virulence of *Past. haemolytica*, Types A and T, for 3-week old lambs were carried out using the intraperitoneal

route of inoculation. Type A strains were derived from cases of enzootic pneumonia in adult sheep and Type T strains from cases of septicaemia in lambs over the age of 6 months. Evidence of considerable virulence was obtained for Type A but not Type T strains. Inoculation of adult sheep with a strain of each Type showed that these animals were highly resistant.

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